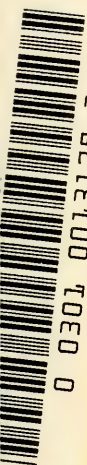


THE LUMINESCENCE OF  
BIOLOGICAL SYSTEMS

Frank H. Johnson





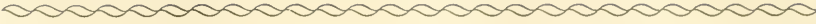


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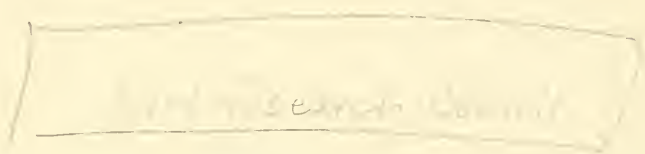







# The Luminescence of Biological Systems

*Proceedings of the Conference on Luminescence March 28-April 2, 1954, sponsored by the Committee on Photobiology of the National Academy of Sciences-National Research Council and supported by the National Science Foundation*



Edited by  
**FRANK H. JOHNSON**  
Princeton University

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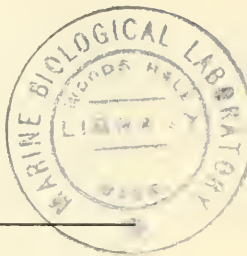


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## Preface

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Luminescence—the emission of “cold light” in the form of bioluminescence, chemiluminescence, fluorescence, phosphorescence, et cetera—has long been a fascinating subject of inquiry. Modern chemical and physical research has greatly advanced the understanding of fluorescence and phosphorescence, particularly of inorganic and of many organic substances; within the past generation, practical applications have become a part of everyday experience, as witnessed by such familiar examples as fluorescent lighting, phosphorescent paints or tapes, and television screens. Luminescence in relation to biological processes is a more difficult and complicated problem, the progress of research has been seemingly slow, and the importance of studies on this aspect has been less obvious. Thus, it has not yet proved possible to obtain luminescent extracts from more than about half a dozen of the myriads of visibly luminescent organisms that are scattered over the phylogenetic tree of animals from protozoa to fishes, as well as over the primitive plant world of bacteria and fungi. Furthermore, basic relationships between photosynthesis, fluorescence, and chemiluminescence of chlorophyll in green plants remain to be clarified, and the full significance of the property of fluorescence in many biological molecules such as riboflavin is yet to be understood.

Within the past several years, however, remarkable progress has been achieved, and broad implications of luminescence in fundamental biological research have become convincingly evident. The way has been paved by an increasing number of investigators, as well as by the ever expanding knowledge in various fields and the introduction of new methods of analysis and measurements. Among our contemporaries, E. Newton Harvey is outstanding for his own extensive and penetrating studies, over a period of more than four decades, as well as for the work he has inspired in his students and colleagues.

Prominent also are the studies led by A. J. Kluyver in the Netherlands, and by Yata Haneda and others in Japan. The luminescence of biological systems has now ripened into a fruitful area of general significance.

Against this background, in brief, the Committee on Photobiology of the National Academy of Sciences-National Research Council, under the chairmanship of Sterling Hendricks and with the support of the National Science Foundation, recognized the mutual advantages of bringing together a group of leading investigators for a critical appraisal of present knowledge, for the first-hand interchange of experiences and ideas, and for the projection of likely approaches to unsolved problems. The conference that resulted was planned to include all aspects of bioluminescence, together with fundamental aspects of chemiluminescence and fluorescence. It was organized by a sub-committee composed of L. R. Blinks, E. N. Harvey, F. H. Johnson (Chairman), W. D. McElroy, and C. E. ZoBell, and it was held at Asilomar, near Pacific Grove, California, March 28–April 2, 1954. The number of participants was restricted, partly because of limitations of available funds, and partly because it was considered that, with the purposes in view, a small group would be most favorable to success. Although the conference was thus restricted, the papers and chief discussions are made available to all who may be interested through the publication of this book.

The first paper, appropriately presented by Harvey, summarizes some important aspects of the present status of our knowledge of bioluminescence and the outlook for further advances. It includes an invaluable tabulation of all the large groups of animals and plants in which there are luminescent representatives, together with typical genera of luminous organisms, their habitat, the availability of histological information about the luminous organ, and the demonstrated biochemical properties of the system, such as the necessity of oxygen or of ATP, the separability of a luciferin and luciferase, the color of fluorescence when present, the color of the bioluminescence, and the susceptibility to inhibition by light.

After the introductory survey, various avenues of approach, from the purely physical to the purely biological, are dealt with comprehensively and in pertinent detail.



In their paper on luminescence spectroscopy, Becker and Kasha deal with the primary step for the utilization of energy by the chlorophyll system, and in particular, the question of which electronic state of chlorophyll is involved in the primary step. Omitting unessential details of the physical theory, they consider chiefly the role of  $n-\pi$ -transitions in the chlorophylls, the importance of intercombinations in these molecules, and the possible interaction of the ethylenic potential function with the electronic transitions in the chlorophylls.

In the first of the two papers that follow, Arnold briefly presents some new data on delayed light production in green plants, together with a hypothesis to account for the observation that light saturation of the delayed light emission occurs at lower intensities than light saturation of photosynthesis. French then gives a summary discussion of fluorescence spectrophotometry of major photosynthetic pigments, including phycoerythrin, bacteriochlorophyll, chlorophylls *a*, *b*, and *c*, protochlorophyll, phycobilins and chlorophylls in the red algae, and a new leaf pigment that was discovered with a fluorescence microscope. An apparatus for automatically plotting the spectral energy distribution of weakly emitting light sources, and its application to the study of pigments in living cells as well as in solution, is described and illustrated in adequate detail.

The kinetic approach to the mechanism of chemiluminescence of the 2,3-dihydrophthalazine-1,4-diones (DPD's or phthalic hydrazides, of which luminol is probably the best known derivative) is stressed in the paper by Wilhelmsen, Lumry, and Eyring. The theory of absolute reaction rates is applied in interpreting the rates of excitation, radiation, and quenching of the molecules involved in light emission. New data are included in regard to the relationships of the reaction to oxygen, and the nature of intermediary compounds and end products is discussed.

Chemiluminescent, fluorescent, and absorption spectra in relation to molecular structure, with particular reference to DPD and naphthoquinone derivatives, as well as dimethylbisacridinium nitrate, are discussed in the joint paper by Spruit and Spruit-van der Burg. The influence of factors such as temperature and pH is included, and the significance of the photochemical inactivation of bioluminescence in

seeking to identify the nature of the light-emitting molecule in living cells is as critically evaluated as present information permits.

The biochemical approach is emphasized in the next four papers, which deal with the luminescent systems of *Cypridina*, the firefly, and luminous bacteria. Tsuji, Chase, and Harvey discuss the most recent chromatographic and electrophoretic experiments which have resulted in highly purified *Cypridina* luciferin and have made it possible to show that such preparations exhibit not only fluorescence but also, in certain solvents, phosphorescence. Changes in the absorption spectrum resulting from oxidation, or from changes in pH, are described. Data on the infrared absorption spectrum are contributed in the discussion by Mason. Products of hydrolysis furnish a partial clue as to the nature of the luciferin, although the structure of the molecule remains to be established.

The biochemistry of firefly luminescence, including the most recent advances, is summarized in the paper by McElroy and Hastings. The role of activators, including ATP, pyrophosphate, and Mn or Mg ions, as well as the action of inhibitors and the influence of variations in concentration of reactants, variations in temperature, pH, oxygen tension, and other factors, is considered in detail. On the basis of the available evidence, a reaction scheme is proposed to account for the control of either steady state or flashing luminescence in both the intact organ and in extracts of the firefly. Additional data on the chemical and physical properties of firefly luciferin are contributed in a brief paper by Strehler, including evidence, from mass spectroscopy of chromatographically purified luciferin, suggestive of a dipyrimidopyrazine nucleus.

The biochemistry and mechanisms controlling the emission of light in extracts of luminous bacteria is treated at some length by Strehler, who recently succeeded in obtaining easily visible luminescence in cell-free preparations of these organisms. The roles of flavine mononucleotide, coenzyme I, and a long chain aliphatic aldehyde, all of which appear to be necessary for a long-lasting, bright luminescence of the enzyme preparation, are discussed. A detailed reaction scheme postulating terminal reactions with a peroxide is presented to account for the available data, including the effects of temperature and hydrostatic pressure. Considerations bearing on this scheme, in particular,

and on the relationships between free energy, activation energy, and emission of visible light in chemiluminescent reactions in general are set forth in discussions by Eyring, Mayer, and Kauzmann, respectively. Further data on the bacterial system, based on considerably purified bacterial luciferase, are contributed in the paper that follows by Hastings and McElroy.

The physical chemistry of activation and inhibition of intracellular luminescence, with special reference to the fundamental action of temperature, hydrostatic pressure, and chemical agents on the reaction rates and equilibria involved in the process of light emission and other biological processes is discussed, apart from the mathematical details of the quantitative theory, in the paper by Johnson. The emphasis is on points of general interest, with a somewhat more detailed discussion of the significance and interpretation of recent data on the kinetics of luminescence in cell-free extracts.

Physiological control of luminescence in animals of varying degrees of complexity, from protozoa and coelenterates to animals with a central nervous system, including worms and fishes, is dealt with by Nicol. Quantitative data on the characteristics of the luminescent response to electrical stimulation in representative types illustrate facilitation, fatigue, and other phenomena familiar in neuromuscular physiology, as well as some effects that are not so generally familiar. Problems in the analysis and interpretation of the physiological control of luminescence in various types of animals are critically examined in the following paper by Buck.

A great diversity of luminescent organisms, terrestrial as well as marine, found in Japan and the Far East is described by Haneda in a comprehensive account that includes first hand observations on distribution, ecological relationships, morphology, histology, physiology, natural history, and other aspects of distinct biological interest. An extensive list of references gives access to the important Japanese literature.

The ecology of marine dinoflagellates, with particular reference to the biological and environmental factors associated with the occurrence of "red water" conditions, is presented in the paper by Ryther. The discussion includes the influence of nutrient requirements, salinity, temperature, and air and water currents. Instances of red water con-

ditions that have been recorded in various parts of the world from 1891 to 1945 are summarized in a table, along with specific organisms involved, the presence or absence of luminescence, the occurrence of mass mortality, and brief notes on the ecological conditions.

The final paper by Haxo and Sweeney deals with the cultivation and some of the physiological characteristics of the photosynthetic, marine dinoflagellate, *Gonyaulax polyedra*, which occasionally gives rise to remarkably vivid displays of luminescence in the coastal waters of southern California.

It is both pleasant and appropriate to record grateful acknowledgment of the assistance rendered in many ways toward the success of the Conference: by the National Science Foundation, the National Academy of Sciences-National Research Council, the members of the sub-committee on arrangements, the management of Asilomar, the authors of the papers and discussion, and the publishers. The editorial task has been lightened in various ways, especially by the prompt and efficient cooperation of the authors and of all others concerned with the publication. Editorial changes in manuscripts were rarely made and they were chiefly for correcting a few obvious errors. The editor must assume full responsibility, however, for the author, genera and species, and subject indexes, as well as for whatever errors, shortcomings, or usefulness may be found in them.

F. H. J.

Princeton, N. J.  
January 1, 1955



Members of the Conference on

## *The Luminescence of Biological Systems*

Asilomar, Pacific Grove,  
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- DR. BEATRICE M. SWEENEY, Scripps Institution of Oceanography, La Jolla, California
- DR. FREDERICK I. TSUJI, Department of Biology, Princeton University, Princeton, New Jersey
- MR. PAUL C. WILHELMSEN, Chemistry Department, University of Utah, Salt Lake City, Utah

CONFERENCE MEMBERS

xi

DR. E. J. FERGUSON-WOOD, Marine Biology Laboratory, Cronulla, New  
South Wales, Australia

DR. CLAUDE E. ZOBELL, Scripps Institution of Oceanography, La Jolla,  
California







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# Survey of Luminous Organisms: Problems and Prospects

E. NEWTON HARVEY

Department of Biology, Princeton University, Princeton, New Jersey



One of the first questions to be settled on discovery of a living luminous organism is whether the species is truly self-luminous, with light from a chemiluminescent system of its own, or whether the light comes from luminous bacteria. The answer is important because light emission from luminous cells is usually associated with coccus-like granules, which look like bacteria, and the existence of symbiotic luminous bacteria in a number of species has been definitely established.

There are in fact three categories of luminous bacteria associated with organisms. In addition to the saprophytic varieties, growing on dead fish or flesh, parasitic bacteria occasionally attack living sandfleas, midges, and caterpillars, giving rise to a luminous disease which is usually fatal to the infected individual. Nevertheless, the animals are active while living, and have been reported as luminous species.

A much more important and widespread type of luminescence results from bacterial symbiosis, common among the squid and the fish. Every individual of a species whose light is associated with symbiotic luminous bacteria must be luminous. In addition, the host often possesses complicated luminous organs in which the symbionts grow. Although the luminous bacteria emit a continuous light, the fish or squid may develop special devices, movable screens, or migrating chromatophores by which the bacterial light can be obscured.

The most striking instances of bacterial symbiosis occur in the Indonesian fish, *Photoblepharon* and *Anomalops*, two genera of the

family Anomalopidae, from the Banda Islands. These fish are characterized by a white spot under the eye, made up of a large group of elongated cells, richly supplied with blood vessels, forming a distinct oval organ in which the symbiotic luminous bacteria grow. The light is continuous but can be cut off—in *Photoblepharon* by a black fold of skin which is drawn over the organ like an eyelid, in *Anomalops* by rotating the organ so that the light shines against the body, rather than out toward the surroundings. Nothing is known of the embryology of these fish, but all individuals are luminous and the bacteria could have entered the light organ from the sea water. There are openings between the region of luminous cells and the environment. Usually fish or squid with symbiotic luminous bacteria possess such open luminous organs, often with a long duct through which the bacteria can be squeezed, as in the fish *Malacocephalus*. Symbiotic luminous bacteria often require a special culture medium for luminescence, whereas saprophytic forms will live on any dead animal.

Table I (column 2, type of light) presents the distribution of all groups (arranged phylogenetically) in which species have been reported as luminous while living. The luminous forms in which bacterial symbiosis ( $B_s$ ) or bacterial parasitism ( $B_p$ ) have been observed, are contrasted with self-luminous forms, marked E or I. In the first column of the table, the habitat of the luminous groups is indicated, whether marine, M, fresh water, F, or terrestrial, T. It will be observed that bacterial symbiosis is most common among the fish. Sometimes it is difficult to distinguish between harmless parasitic luminous bacteria and the symbiotic variety. Certain squid usually contain luminous bacteria in a special gland, but in some individuals no light is visible on dissecting and opening the gland. In such cases how can one decide between symbiosis and harmless penetration by luminous bacteria? It was once supposed that all bioluminescences were of bacterial origin, but it is now quite certain that symbiosis among luminous animals is not nearly as widespread as had been supposed. The reader is referred to the work of Pierantoni (1936, 1939)\* and of Buchner (1953) for a discussion of this field.

\* Literature prior to 1951 will be found in the bibliography of Harvey in *Bioluminescence*, Academic Press, New York, 1952. Only later references are given in this paper.



In addition to the genera listed in the table, occasional reports of luminous species are to be found in the literature for the following groups: Ascomycetes (*Xylaria*); Phallales (*Illeodictyon*, *Kalchbrennera*); Turbellaria (*Monocelis*); Polychaeta (*Nereis*, *Polyopthalmus*); Polyzoa (*Membranipora*, *Flustra*, *Electra*, *Acanthodesia*<sup>\*</sup>); Chaetognatha (*Sagitta*); Pteropoda (*Creseis*, *Styliola*, *Clio*, *Cavolinia*); Prosobranchiata (*Pterotrachea*, *Tonna*); Octopoda (*Cirrothauma*, *Eledonella*); Pycnogonida (*Colossendeis*); Araneae (Spiders); Isoptera (termites); Lepidoptera (*Arctia*); Asteroidea (*Brisingia*); Ascidiacea (*Ciona*); Chimaerae (*Chimaera*); Teleostomi (*Parehippus*, *Ruvettus*, *Exocetus*, *Xenodermichthys*, *Halosaurus*, *Bassozetus*, *Leucicorus*, *Macrurus*, *Lamprogrammus*, *Mixonus*, *Malthopsis*, *Ipnops*). Nothing is known of the nature of the light emission and confirmation of luminosity is to be desired.

The title of this conference, "The luminescence of biological systems," suggests that self-luminous organisms are the principal ones to be considered and that the chemistry of the various luminescent systems should receive first attention. How may the more than 70 quite different groups of luminous organisms listed in Table I be arranged on such a basis? It is always difficult to classify a subject, because those familiar with the field may disagree regarding the proper basis for classification, and because of the usual exceptions to every rule. One basis for characterizing luminescent organisms depends on whether the light emission takes place within the cell, intracellular, I, or the luminous substances are secreted to the outside, extracellular, E. The groups belonging in these categories are indicated in Table I, column 2.

Perhaps a more fundamental grouping, for which there can be little criticism on biological grounds, is (1) luminous plants and (2) luminous animals. This classification not only reflects the fundamental difference in chemistry in the animal and vegetable worlds, but is also particularly cogent because in the plant kingdom, whose only luminous representatives are bacteria and fungi,<sup>†</sup> light production is

<sup>\*</sup> See K. Kato, *Dobutsugaku Zasshi*, **59**, 9–10, 1950.

<sup>†</sup> The chemiluminescence following photosynthetic processes, invisible to the dark adapted eye, which has been described by Strehler (1951) and Strehler and Arnold (1951) is in a different category from bioluminescences.

continuous and independent of stimulation. The luminescence intensity varies only with environmental changes, such as temperature, pH, and salt content of the medium. Among animals, no light appears until the luminous region is excited in some way—by nerves or directly by mechanical, electrical, and chemical stimulation. The resulting luminescence is momentary or of short duration. In a few fish (*Porichthys*, *Echiostoma*, *Maurolicus*, *Argyropelecus*) light emission of photophores is hormone (epinephrin) controlled and the light lasts a longer time than in the case of direct nerve stimulation.

No bacteria or fungi are known, which can be stimulated to luminescence, but a few animals, whose light is not due to luminous bacteria, can luminesce continuously. These exceptions to the general rule are found in the diplopod millipede *Luminodesmus* (Davenport, Wootton, and Cushing, 1952), and in various stages of firefly development. The firefly egg and the pupa are continuously luminous, as are adult cream-colored wingless females of the related beetle, *Phengodes*. The luminous organs of the females may remain brightly luminous for days, with no change in intensity. *Luminodesmus* is continuously luminous, with no voluntary control, and luminous bacteria appear to be absent, at least they have not been demonstrated.

Light emission of both larval and adult luminous organs of the firefly is controlled by nerves, but during the pupal stage, the larval organ ceases to function and the adult lantern of the firefly develops as a wholly new structure, in a new position on the abdominal segments. It is completely reconstituted by luminous wandering cells (not subject to nerve stimulation) whose light shines through the chitinous integument.

Protozoa respond directly to stimulation, usually to mechanical disturbance resulting in the "phosphorescence of the sea," but metazoan\* luminous cells are supplied by nerves, and reflex luminescence

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All other reported cases of luminescence of plants are due to reflection or in the case of marine algae, due to luminous organisms growing on the seaweed. Luminous dinoflagellates or peridineeae are regarded as animals for this discussion because they luminesce only on stimulation.

\* Among sponges no electrical excitation of luminescence could be elicited, but response occurred on rubbing the sponge (Harvey, 1921). Sponges do not possess nerves.

occurs, either as a result of secretory nerve stimulation in forms with extracellular luminescence, or from an effect similar to the action of a motor nerve on a muscle, from the impulse of what might be called a "luminor nerve," rather than a "luminous nerve." The recording of light from stimulation of nerves or luminous tissue is a field of special interest, first studied with luminous beetles (Snell, 1931, 1932; Brown and King, 1931; Harvey, 1931), and recently greatly extended by Nicol (1952, 1953), and by Harvey and Chang (1954). The results will be considered in detail in this volume.

Another field of considerable interest which should be investigated with modern equipment and microelectrode technique is the electrical change accompanying luminescence. A beginning has been made by Hasama (1939-1944), who has published "electroluminograms" representing the electrical potential change during luminescence of the glowworm, the firefly, the worm *Chaetopterus*, the mollusc *Plocamophorus*, and the pennatulid *Cavernularia*.

No physiological or biochemical investigation can be considered adequate without a knowledge of the histology, particularly the fine structure of luminous cells or tissue. The extent to which this is known among various luminous groups and the regions for further research are indicated in Table I, column 3. That the light generally arises from cellular granules is a most significant and important observation deserving further study.

Bacterial and fungal luminescence, in which steady state conditions determine the amount of photogenic material undergoing change, are probably the most difficult to analyze from the chemical standpoint. Precursors of the photogen, as well as reaction products and various enzyme systems, may be expected to influence the continuous luminescence. A continuation of the kinetic studies on bacterial luminescence initiated by Johnson and collaborators (see 1954) is much to be desired. It is also to be expected that extracellular luminescent systems should be different from intracellular ones as in bacteria or those of the firefly. In the firefly, the cell reactions are designed for building up a supply of photogen which suddenly reacts with a flash of light following stimulation. Like bacteria, the firefly luminous cells must contain the precursors of the photogen and also the reaction products, as well as various enzyme systems.



In an organism with extracellular luminescence such as the ostracod crustacean *Cypridina*, gland cells are filled with the finished secretion to be extruded to the sea water by the contraction of muscles. In such a case we may expect the principal luminous substances directly concerned with light production to be present, with a minimal amount of precursor. The reaction products form in the sea water. *Cypridina* does possess the simplest chemical system thus far investigated, one in which the only recognized substances are oxygen, a heat stable, dialyzable, oxidizable substance, luciferin and a thermolabile enzyme, luciferase, specific for the light-emitting oxidation of luciferin in aqueous solutions. In the *Cypridina* gland, the luciferin and luciferase are manufactured in separate gland cells. Since the chemistry of luminous bacteria, of the firefly, and of *Cypridina* is individually represented in this conference, a discussion of the accessory factors involved in bacterial and firefly luminescence will be found in the appropriate chapter. Studies on luciferase kinetics, similar to those made by Chase (1946-52) with *Cypridina* luciferase, should be extended to other organisms.

The ability to demonstrate the luciferin-luciferase reaction according to the method described in the explanation of Table I is presented in column 5. In addition to *Cypridina*, fireflies, and bacteria, only the marine fireworm of Bermuda (*Odontosyllis enopla*), the deep sea shrimp (*Systellaspis* and *Heterocarpus*), and the fresh water limpet\* (*Latia neritoides*), of New Zealand, have been reported as positive for a luciferin-luciferase reaction.

Although firefly biochemistry will be considered in another chapter, it must be pointed out that in the firefly luminescent system, adenosine triphosphate (ATP) is an important accessory substance. This can be demonstrated by the fact that a cold water extract of the luminous tissue of the firefly allowed to stand until all light disappears, will again luminesce when ATP is added (McElroy, 1947, 1951). A systematic test of similar ATP action in other luminous organisms has been made by Harvey and Haneda (1952) and Haneda and Harvey (1954) and is included, together with later tests, in Table I, column 6. The importance of ATP in luminescent reactions appears to have

\* According to Bowden (1950).



been definitely established only among the elaterid and lampyrid beetles.

The absence of an ATP reaction does not necessarily mean that ATP plays no part in light production (McElroy and Harvey, 1951). It may indicate that other components of the luminescent system are lacking and that further analysis may be necessary to designate the complete system. Similarly, the absence of a luciferin-luciferase reaction may indicate that accessory substances are lacking or that luciferin and luciferase are particularly unstable substances in the group of luminous organisms tested.

In view of the recent work (Strehler, 1953; Strehler and Cormier, 1953; McElroy, Hastings, Sonnenfeld, and Coulombre, 1953; Strehler, Harvey, Chang, and Cormier, 1954) on bacterial luminescence, where the luminous system is complicated by accessory substances such as long chain aldehydes, as well as in firefly luminescence requiring high energy phosphates, a redefinition of luciferin becomes necessary (Harvey and Tsuji, 1954). It is no longer sufficient to claim that luciferin is present in a boiled extract of luminous tissue, whereas the dark cold water extract contains luciferase. McElroy has demonstrated that dark cold water extracts of firefly (*Photinus pyralis*) lanterns emit no light when purified *pyralis* luciferin is added, but do luminesce with ATP. Therefore the cold water extract (luciferase) lacks ATP instead of luciferin. ATP is the limiting factor under these conditions.

Rather than placing the emphasis on the limiting factor, or on heat stability or dialyzability, as has been done previously, light emission should be the criterion for luciferin. In the case of luminous organisms requiring dissolved molecular oxygen for luminescence, luciferin may properly be defined as the oxidizable substance supplying molecules capable of absorbing enough excess energy from a chemical reaction to emit in the visible region. Such a definition implies that some form of luciferin molecule—either free base or acid, either dissociated anion or cation, in reduced or oxidized form, either free or combined with protein, like a prosthetic enzyme group—can pick up the energy of the oxidative reaction in which it is involved. Such a definition does not mean that luciferin is the same substance in different luminous animals, nor does it necessarily designate luciferin molecules themselves as the ones which emit, but it does imply that a related mole-

cule, such as a luciferin-luciferase combination, or an oxidized luciferin molecule, or a molecule of an intermediate step, is the emitter. The actual molecule emitting might be designated the "photogen."

It has long been recognized that a substance whose molecules are readily excited to fluoresce by the energy of radiation is most likely to be chemiluminescent from the energy of a chemical reaction. Bacterial luciferin, firefly luciferin and *Cypridina* luciferin are all fluorescent, and it was early observed (Harvey, 1925) that fluorescence of the light organs of luminous animals is a widespread phenomenon. The fluorescence of ctenophore luminous organs is particularly noticeable immediately after the bioluminescence has ceased (Harvey, 1925, 1926). The distribution of marked fluorescence in the luminous organs of various groups is given in column 8 of Table I.

In addition to bacteria, *Cypridina*, and fireflies, the only other luminous system which has received chemical attention in recent time is that of earthworms, studied by a group of Czech investigators, Komarek, Backovsky, and Wenig (see Wenig, 1946). They have demonstrated the presence of riboflavin, not flavin phosphate or flavin adenine dinucleotide (Wenig and Kubista, 1949), in the yellow lymphocytes of the luminous earthworm *Eisenia submontana*, as well as in those of a nonluminous form, *E. foetida*. The luminous lymph of *E. submontana* fluoresces yellow-greenlike riboflavin until the bioluminescence has disappeared, at which time the fluorescence color changes to blue, that of lumichrome. A corresponding change in the yellow-green fluorescence of the nonluminous lymph of *E. foetida* does not take place. Consequently the Czech workers first postulated that the bioluminescence of the earthworm is connected with a change from riboflavin to lumichrome, a reaction which does not occur in the nonluminous species. Later they state that the molecules of riboflavin are believed to be "absorbed in an oriented layer on the surface of granula of lipoid character . . . [and] the activation energy which brings them into an excited state is probably derived from an oxidative reaction in which molecular oxygen takes part."

It is interesting to note that the luminous granules of the earthworm are yellow. The luciferin of *Cypridina* is also yellow (although its luminescence is blue), and a yellow color has been observed associated with luminous cells in at least seven additional groups—Hydrome-

dusae, Polychaeta (Tompteridae and Terebellidae), Nudibranchia, Copepoda, Chilopoda, Lampyrid fireflies, and Macrurid fish.

In many other luminous organisms a yellow color cannot be established with certainty although a faint yellow would be difficult to detect. Of particular interest is the cephalic luminous organ of the railroad worm (*Phrixothrix*), whose bioluminescence is a bright red, quite similar to the fluorescence of hematoporphyrin and to the chemiluminescence of metal porphyrin compounds. One might expect to find a faint red tinge in the organ from a porphyrin, or a red fluorescence, but the tissue appears quite colorless to the eye and is non-fluorescent in ultraviolet or in yellow to violet light (Harvey, 1944, 1945). It is certain that no red color screen is involved in the red luminescence of *Phrixothrix*.

Another method of grouping luminous organisms involves the necessity or non-necessity of oxygen for luminescence. The author (1926), in a systematic study of oxygen requirements for luminescence of various groups, was amazed to find that the ctenophores *Beroë* and *Eucharis*, the scyphomedusan *Pelagia*, and the radiolarians, *Thalassicola* and *Colozoum*, require no dissolved oxygen for light production. The result has been confirmed for another ctenophore, *Mnemiopsis* (Harvey and Korr, 1938), and for the hydromedusan *Aequorea* (Anderson, private communication, 1939). It should be emphasized that it is the luminescent system of extracts of various ctenophores which emits light without dissolved oxygen, whereas the ability to stimulate a ctenophore to luminescence through nerves may be lost in absence of oxygen (Chase, 1941). The relation between oxygen pressure and luminescence intensity should be studied for all organisms (see Hastings, 1952, 1953).

Another basis for classification of luminous groups depends on their relation to light, whether light inhibits the luminescence or not. Such a division might involve inhibition of a nerve-stimulating mechanism, or a photochemical action on the chemiluminescent system. Such an effect of ultraviolet light and photosensitized visible light on *Cypridina* luciferin will be referred to in the chapter on *Cypridina* chemistry. The most important and best known case of light inhibition is to be found among ctenophores. They do not luminesce in sunlight or strong electric light but regain the ability after some twenty minutes in the



dark. The effect of strong light is on the luminescent system. Weak light will inhibit the excitation mechanism (Moore, 1924). No day-night rhythm of luminescence has been established in the ctenophore, *Mnemiopsis*, but among dinoflagellates, a day-night rhythm has been described. The complicated details of light inhibition have been described in *Bioluminescence* (Harvey, 1952), and the action on various groups is summarized in Table I, column 7.

This survey of luminous organisms has indicated certain well-defined biochemical groups, but there are many others about which too little is known to attempt a logical separation. Luminous animals are scattered over the evolutionary tree from Protozoa to fish without any indication of direct evolutionary descent. One species of a genus may be luminous and another not. One variety of a fungus may be luminous and another not. The existence of nonluminous mutants of luminous bacteria has been known since the work of Beijerinck (1912). The author (1932, 1953) has taken the viewpoint that luminescence has arisen independently in various phyla of the animal kingdom, probably from some slight change in chemical systems common to cells in general, most likely the cell-respiratory systems.

There is every evidence that the luciferins of fireflies, bacteria, and Cypridinae are chemically quite different and must be prefixed by the word firefly luciferin, bacterial luciferin, etc., in order to designate them. The bioluminescence emission spectra of various organisms range in the wavelength regions of red to violet, just as do the chemiluminescence spectra of quite unrelated organic compounds—metal porphyrins, red; pyrogallol, yellow; dimethyldiacridinium nitrate, green; 3-aminophthalic hydrazide, blue luminescence. Substituted groups on these compounds will change the chemiluminescent intensity markedly and the maximum wavelength of emission slightly, but thus far spectral energy curves have given little clue to chemical structure.

It must be emphasized that small shifts in maximum emission of bioluminescences have little meaning, since the presence of absorbing pigments in cells and the phenomenon of differential absorption may change the emission spectrum of any luminous animal. The latter effect is particularly well seen in Spruit-van der Burg's (1950) study of the relation of density of suspension to bacterial luminescence emission.

The maximum wavelength changes from 500 to 470 millimicrons as the suspension density changes from 40 to 8 arbitrary units.

The various species of luminous bacteria do possess different maxima in the blue region when measured in dilute suspension (Spruit-van der Burg, 1950). The light of luminous bacteria looks green to the eye when the intensity is sufficiently high to involve color vision, no doubt because the spectral energy curve is skewed, with greatest energy on the long-wavelengths side of the maximum around 480 millimicrons. In this respect bacterial luminescence differs from that of the blue *Cypridina* luminescence, whose maximum emission is about 480 millimicrons, but the spectral energy curve is more narrow and symmetrical. Because of the above considerations, bioluminescent emission spectra are not too significant. Nevertheless, as a guide to previous investigations, the measured maximum wavelengths and the reported color of the light of various groups have been collected in Table I, column 9.

It would be ideal if luminous groups could be separated on the basis of the chemiluminescent systems involved, primarily depending on the structure of luciferin. At the present time that is not possible, but certain organisms can be classed together with reasonable certainty on the basis of similar chemical behavior. There is little doubt but that the fungi and the bacteria contain similar luminescent systems, although the role of flavins in fungal luminescence is as yet not demonstrated.

So little is known of the biochemistry of luminescence of the Protozoa that it is very difficult to predict a similarity with any of the known chemiluminescent systems. *Noctiluca* and smaller forms can often be obtained in enormous numbers and should offer good material for chemical research, except that the percentage of luminous substance is undoubtedly small. In *Noctiluca*, light is always associated with minute granules, which flash on stimulation and emit a steady glow under conditions of injury to the cell. No form is better adapted for cell physiological studies, particularly of the excitation process. *Noctiluca* is an ideal organism for the investigation of the all-or-none law, time relations of the flash, repetitive stimulation, fatigue, conduction of local excitation, etc., in a single cell. Among other advantages, *Noctiluca* is large and nearly spherical, 0.5–1.0 mm in diameter, pos-

sessing a single nucleus and a sap vacuole into which substances may be injected or sap withdrawn. It also possesses a flagellum and a tentacle, whose movement can be studied. In addition there are visible protoplasmic changes on electrical stimulation.

There are also interesting osmotic relations in *Noctiluca*, connected with a specific gravity less than that of sea water. The low density is due to a definite salt content, not to oil droplets. Permeability, movement, and light emission can all be studied together in this single cell—a most unusual situation. The relation between oxygen pressure, temperature, hydrostatic pressure, pH, salts, drugs, etc., and light emission and movement should be worked out in detail.

The smaller dinoflagellates are not as favorable material as *Noctiluca* for the cell physiologist. However, the relation between oxygen pressure and light emission should be studied carefully. The Radiolaria do not require oxygen for luminescence, and this peculiarity may be more widespread among Protozoa than is now realized. Day-night rhythms of luminescence might be carefully tested in all species. Should it prove feasible to culture luminous Protozoa in large numbers under laboratory conditions, they should be as valuable for biochemical work as the luminous bacteria.

The great groups of luminous coelenterates and ctenophores behave alike in many ways. The luminous tissue can be ground in sea water to a dark extract that will give a brilliant light when added to fresh water. Luminescence comes from granules which dissolve with light emission as a result of treatment with many agents, such as saponin, which cause the cytolysis of cells. The chemistry of these forms is completely unknown and offers a virgin field for investigation, since material can no doubt be prepared by modern methods of freeze-drying. As we have seen, in some species dissolved oxygen is not necessary for luminescence and in other species photochemical changes in the luminescent system prevent luminescence after the animal is exposed to daylight.

In addition, the physiology of luminescence in medusae, pennatulids, and ctenophores presents many problems connected with excitation of light by luminor nerves and reflex transmission of impulses. A beginning has been made by the work of Parker (1920), Buck (1953), and Harvey and Chang (1954), but much more needs to be done. The

wave of light which spreads over a pennatulid colony stimulated at one point was observed by L. Spallanzani in 1783 and has excited the interest of investigators ever since.

Marine annelids are much more diverse than the coelenterates in methods of light production. Some secrete from epidermal glands, some exhibit intracellular luminescence. The color of the luminescence varies from yellow to blue. A luciferin-luciferase reaction has been demonstrated in *Odontosyllis*, and this worm offers special advantages for chemical study, as it swarms in large numbers at Bermuda and other places in relation to phases of the moon. In annelids, the nervous control of luminescence is particularly favorable for study and has been thoroughly investigated by Nicol in *Chaetopterus* (1952) and in polynoid worms (1953).

Earthworms are a favorable group for chemical studies. Reference has already been made to the Czech investigations. In addition to the part played by flavins in luminescence, attention should be particularly directed to the fact that one species of the genus *Eisenia* may be luminous and another species not, even though closely related. What is lacking in the nonluminous species? Cross breeding should be attempted. Study of such intercrosses between varieties of the fungus *Panus stipticus* has already given interesting results (Macrae, 1942).

Among molluscs, very little has been added to the chemistry of light production since the work of Dubois, which is summed up in his 1928 article in Richet's *Dictionnaire de Physiologie*, Vol. X. *Pholas* is extraordinarily favorable for chemical work, and should be attacked by modern methods.

Self-luminous squid offer many species with complicated luminous organs for physiological work, but they are mostly deep sea forms, rare and unfavorable for chemical studies, with the exception of *Watasenia scintillans*, which breeds in enormous numbers during April-June in Toyama Bay on the western coast of Japan. Nerve and possible hormone control of the lighting mechanism presents an untouched field for investigation.

Among crustacea, ostracods, copepods, mysids, and decapod shrimp all produce an external secretion, often in great abundance. It might be expected that luciferin from one species would react with luciferase

from another species of all the groups, but such is not the case. No luminescence appears when luciferin of *Cypridina* is mixed with solutions which should contain luciferase from copepods, or from decapods, and vice versa. Only if the luciferin is from another genus of ostracods (*Pyrocypris*) will it react with *Cypridina* luciferase, or will the reverse "cross" be positive. This specificity of the luciferin-luciferase reaction is widespread in the animal kingdom. The chemistry of all the crustacea with external luminous secretions should be investigated in detail.

Crustacea with scattered photophores present problems of physiological interest, but do not appear to be favorable for chemical work, because of the small volume of luminous material. It is possible that the photophores are hormone controlled. This relation and nerve control of lighting should be carefully investigated.

Among myriapods, the facts concerning light production of the diplopod *Luminodesmus* has been presented by Davenport, Wootton, and Cushing (1952). Chilopods produce an external secretion in great abundance, which should be intensively studied. The chemistry may be similar to that in earthworms, but not enough is known to make a comparison. If luminous centipedes could be bred in captivity they should be highly favorable forms for biochemical work.

Among insects, apart from the Coleoptera, whose luminescence has been much investigated, the light of Collembola, Diptera (see Kato, 1953), and the controversial genus *Fulgora* should be studied in great detail—morphology, histology, physiology, and chemistry. Very little is known of light production in these rather uncommon luminous insects.

That fireflies have been of great value for biochemical work is apparent from the report presented in another section. The rapid flash of a firefly still remains an unsolved problem in insect physiology. Behavior studies in different species of fireflies in regard to the use of the light (see Buck, 1937, 1948), present a promising field for the ecologist. The meaning and mechanism of synchronous flashing of tropical fireflies is still a mystery. Finally the spectral energy distribution of the light of various species should be explained. Since Coblenz' monograph of 1912, little has been done to determine whether the range in color of the light from orange to yellow green



is due to a characteristic of the chemiluminescent reaction or to absorption by pigments. The extreme range of color appears in the railroad worm *Phrixothrix* with both yellow lights and also a bright red light in the head, which is not connected with any red pigment detectable with the eye.

The ophiuroids, despite considerable histological study, present many unsolved problems of physiological and biochemical nature, and the same may be said for the balanoglossids and the tunicates. Luminous species are abundant among surface marine organisms and are easily obtainable.

The considerable number of fish whose light is not due to luminous bacteria are in the same category as the squid. They are mostly deep sea forms with photophores, not favorable for chemical work but presenting many physiological problems in connection with nerve and hormone excitation. They deserve intensive investigation, whenever the occasion allows. In fact it seems certain that luminous organisms as a whole can supply much information important for an understanding of fundamental processes in every field of biology.

TABLE I

Key to column heads:									
1	Habitat	4	Oxygen necessary	5	Luciferin-luciferase reaction	7	Inhibition by light	8	Fluorescence color
2	Type of light	6	ATP reaction	3	4	5	6	7	8
3	Knowledge of histology	1	2	3	4	5	6	7	8
Group and Typical Luminous Genera									
	Bacteria ( <i>Photobacterium</i> , <i>Achromobacter</i> , <i>Vibrio</i> )	MF <sup>c</sup>	I	±	+	+	—	—	+
	Fungi basidiomycetes ( <i>Panus</i> , <i>Omphalia</i> , <i>Pleurotus</i> , <i>Polyporus</i> , etc.)	T	I	±	+	—	—	—	—
	Radiolaria ( <i>Thalassicola</i> , <i>Sphaerocozum</i> , <i>Collazoum</i> , <i>Myxosphaera</i> , <i>Collosphaera</i> )	M	I	±	—	—	—	—	—
	Dinoflagellata ( <i>Noctiluca</i> , <i>Ceratium</i> , <i>Gonyaulax</i> , <i>Gymnodinium</i> , <i>Pyrocystis</i> , etc.)	M	I	±	+	—	—	+	—
	Porifera ( <i>Grantia</i> )	M	I?	—	—	—	—	—	—
	Hydroidea								
	Hydroids ( <i>Campanularia</i> , <i>Obleia</i> , <i>Aglaophenia</i> , etc.)	M	I?	—	—	—	—	—	—
	Hydromedusae ( <i>Aequorea</i> , <i>Halistaura</i> , <i>Phialidium</i> , etc.)	M	I?	—	—	—	—	—	—
	Siphonophora ( <i>Diphyes</i> , <i>Hippopodius</i> , <i>Agalma</i> , etc.)	M	I?	—	—	—	—	—	—
	Scyphomedusae ( <i>Præglia</i> , etc.)	M	E?	±	—	—	—	—	—
	Pennatulacea ( <i>Cavernularia</i> , <i>Pennatula</i> , <i>Renilla</i> , <i>Pteroides</i> , etc.)	M	I	±	+	—	—	—	—
	Gorgonacea ( <i>Ceratois</i> , <i>Prinnois</i> , etc.)	M	?	—	—	—	—	—	—
	Ctenophora ( <i>Mnemiopsis</i> , <i>Beroë</i> , <i>Pleurobranchia</i> , <i>Bolinopsis</i> , <i>Eucharis</i> , etc.)	M	I	±	—	—	—	+	Greenish



Nemertinae ( <i>Emplectonema</i> )	M	I	+			Bluish white
Polychaeta						
Polynoinae ( <i>Acholoë</i> , <i>Polynae</i> , <i>Harmothoë</i> , etc.)	M	I	+	+	—	Yellowish
Alciopidae ( <i>Calazonella</i> , <i>Corynocephalus</i> , etc.)	M	I	—	+	—	Bluish
Tomopteridae ( <i>Tomopteris</i> )	M	I?	±	—	—	Yellowish
Syllidae ( <i>Odonosyllis</i> , etc.)	M	E	±	+	—	Bluish
Eunicidae ( <i>Onuphis</i> )	M	I	—	—	—	Bluish green
Chaetopteridae ( <i>Chaetopterus</i> , <i>Mesochaetopterus</i> , etc.)	M	E	+	+	—	Bluish
Cirratulidae ( <i>Cirratulus</i> , <i>Heterocirrus</i> , etc.)	M	E	+	—	—	Yellow-green
Terebellidae ( <i>Thalepus</i> , <i>Polycirrus</i> )	M	E	+	+	—	Bluish
Oligochaeta ( <i>Microscolex</i> , <i>Eiscenia</i> , <i>Pontodrilus</i> , <i>Oetochaeta</i> , etc.)	T <sup>a</sup>	E	+	+	—	Yellow-green (550)
Nudibranchia ( <i>Placamopherus</i> , <i>Phyllirhoë</i> , <i>Kaloplacamus</i> , etc.)	M	E <sup>w</sup>	+	—	—	Bluish white
Pulmonata						
Ariophantidae ( <i>Dyakia</i> )	T	I	—			Bluish
Latidae ( <i>Latia</i> )	F	E	+	+	—	Bluish green
Prosobranchia ( <i>Planaxis</i> )	M	I	—	—	—	Bluish
Bivalvia ( <i>Pholas</i> , <i>Rocellaria</i> )	M	E	+	+	—	Greenish blue
Vampyromorpha ( <i>Vampyroteuthis</i> )	M	I	+	—	—	
Decapod squid <sup>i</sup>						
Oegopsida ( <i>Watasenia</i> , <i>Lycoteuthis</i> , <i>Histioteuthis</i> , <i>Calliteuthis</i> , <i>Chiroteuthis</i> , <i>Cranchia</i> , etc.)	M	I	+	+	—	Bluish <sup>j</sup> or yellowish
Myopsida ( <i>Unoteuthis</i> , <i>Rondeletia</i> , <i>Sepioida</i> , <i>Spirula</i> , etc.)	M	B <sub>s</sub> or B <sub>p</sub>	+	+	—	Bluish <sup>k</sup>
Myopsida ( <i>Heteroteuthis</i> , <i>Stoloteuthis</i> ) <sup>a</sup>	M	E	+	+	—	Yellowish
Ostracoda ( <i>Cypridina</i> , <i>Pycrocypris</i> , <i>Conchoecia</i> , etc.)	M	E	+	+	+	Blue (480—470)

TABLE I (Continued)

Group and Typical Luminous Genera	1	2	3	4	5	6 <sup>a</sup>	7	8	9 <sup>b</sup>
Copepoda ( <i>Pleuromma</i> , <i>Leuckartia</i> , <i>Heterochaeta</i> , <i>Oncaea</i> , etc.)	M	E	±	+	—		— + DN	Greenish	Greenish <sup>c</sup>
Isopoda ( <i>Megalligia</i> , <i>Porcellio</i> )	MT	B <sub>p</sub>		+					Bluish
Amphipoda									
Gammaridea ( <i>Talitrus</i> , <i>Orchestia</i> , etc.)	M	B <sub>p</sub>		+					Bluish
Hyperideia ( <i>Scypholanceola</i> , <i>Strepsia</i> , <i>Para- pronoë</i> )	M	I?	±						Greenish yellow <sup>m</sup>
Mysidacea ( <i>Gnathophausia</i> , <i>Mysis</i> , <i>Siriella</i> , <i>Gas- trosaccus</i> )	M	E	±						Greenish
Schizopoda ( <i>Nyctiphanes</i> , <i>Euphausia</i> , <i>Nemato- sacalis</i> , etc.)	M	I	+		—			Greenish?	Bluish
Decapod shrimp ( <i>Scirgastes</i> , <i>Hoplophorus</i> , etc.)	M	I	+		—				Greenish yellow
( <i>Systellaspis</i> , <i>Plesiopenaeus</i> , <i>Heterocarpus</i> , etc.)	M	E	+		+				Bluish
( <i>Xyphocaridina</i> )	F	B <sub>p</sub>		+					Bluish
Diplopoda ( <i>Luminodesmus</i> , <i>Spirobolletus</i> )	T	I B <sub>p</sub> ?	—	+	—			Blue-green	Greenish white
( <i>Trigonotulus</i> )	T	B <sub>p</sub>							
Chilopoda ( <i>Stigmatogaster</i> , <i>Orya</i> , <i>Geophilus</i> , <i>Sco- liophanes</i> , etc.)	T	E	+	+	—			Bluish?	Blue-green
Collembola ( <i>Neanura</i> , <i>Onychiurus</i> , etc.)	T	I <sup>n</sup>	—						Blue-green or greenish yellow
Orthoptera ( <i>Gryllotalpa</i> )	T	B <sub>p</sub> ?	—						Greenish white
Ephemeroidea ( <i>Cacnis</i> , <i>Teloganodes</i> )	T	B <sub>p</sub> ?	—						Pale blue
Hemiptera ( <i>Fulgora</i> )	T	?	—						White
Diptera ( <i>Cerophatus</i> , <i>Platypira</i> , <i>Arachnocampa</i> , etc.)	T	I	±		—				Bluish
( <i>Chironomus</i> )	T	B <sub>p</sub>							Greenish
Lepidoptera ( <i>Mananestra</i> , <i>Agrotis</i> larvae; <i>Astero- scopus</i> adult)	T	B <sub>p</sub>							Bluish

Hymenoptera ( <i>Camponotus, Iridomyrmex</i> )	T	B <sub>p</sub> ?	—	Greenish yellow Orange (565), yellow (570), or green (550) Yellow-green
Coleoptera				
Lampyridae ( <i>Photinus, Photuris, Luciola, Lam- pyris</i> , etc.)	TF	I	+	Greenish <sup>a</sup> yellow +
Phengodidae ( <i>Phengodes</i> , etc.)	T	I	+	Yellowish?
Rhagophthalmidae ( <i>Diopltoma</i> )	T	I	—	Green
Drilidae ( <i>Diplocladon</i> )	T	I	—	Greenish blue
Drilidae ( <i>Phrixothrix</i> )	T	I	+	Red and yellow-green
Elatidae ( <i>Pyrophorus</i> , etc.)	T	I	+	Greenish and orange (518 & 530)
Ophiuroidea* ( <i>Amphiuva, Ophiocolex, Opiopsila</i> , etc.)	M	I	+	Green
Enteropneusta ( <i>Ptychodera, Balanoglossus</i> )	M	E	+	Yellow-green
Tunicata ( <i>Pyrosoma, Appendicularia, Salpa</i> , etc.)	M	I or B <sub>s</sub> ?	+	—
Elasmobranchii ( <i>Etmopterus, Lamargus, Centro- syllium</i> , etc.)	M	I	+	Bluish or greenish
Teleostomi				Various colors
Macrouridae ( <i>Malacocephalus, Coelohynchus</i> , etc.)	M	B <sub>s</sub>	+	Greenish or white
Gadidae ( <i>Physiculus, Lotella</i> )	M	B <sub>s</sub>	+	Greenish blue (510)
Monacanthidae ( <i>Monacanthus</i> )	M	B <sub>s</sub>	+	Greenish blue
Anomalopidae ( <i>Photoblepharon, Anomalops</i> , <i>Kryptophaneron</i> )	M	B <sub>s</sub>	+	Weak bluish Greenish blue
Acropomatidae ( <i>Acropoma</i> )	M	B <sub>s</sub>	+	Bluish white
Leiognathidae ( <i>Leiognathus, Gazzo, Scurator</i> )	M	B <sub>s</sub>	+	Bluish white
Serranidae ( <i>Apogon</i> )	M	I?	+	Bluish green
Ceratioidae ( <i>Dolopichthys, Ceratius, Linophryne</i> , etc.)	M	B <sub>s</sub> ?	+	Bluish green or purplish

TABLE I (Continued)

Group and Typical Luminous Genera	1	2	3	4	5	6 <sup>a</sup>	7	8	9 <sup>b</sup>
Saccopharyngidae ( <i>Saccopharynx</i> )	M	B <sub>2</sub> ?	—						White
Batrachoididae ( <i>Porichthys</i> , etc.)	M	I	+						White
Stomatidae ( <i>Echiostoma</i> , <i>Gonostoma</i> , <i>Maurolepis</i> , <i>Polyipnus</i> , <i>Argyroplecus</i> , <i>Chauliodus</i> , <i>Stomias</i> , etc.)	M	I <sup>u</sup>	+		—	—	—	Greenish <sup>v</sup>	Yellowish to greenish or bluish
Myctophoidae ( <i>Myctophum</i> , <i>Diaphus</i> , <i>Lampanyctus</i> , <i>Neoscopelus</i> , etc.)	M	I	+		—	—		Greenish	Bluish or greenish

<sup>a</sup> No luminous bacteria could be demonstrated by Haneda in *Stoloteuthis*.

<sup>b</sup> Numbers in parentheses, maximum wavelength in millimicrons.

<sup>c</sup> Some luminous bacteria live on the flesh of terrestrial animals.

<sup>d</sup> Inhibition observed by Heymans and Moore (1923, 1924).

<sup>e</sup> ATP reaction positive in *Renilla kolkleri* and negative in *R. reniformis*.

<sup>f</sup> In *Renilla*, inhibition and no inhibition have been reported.

<sup>g</sup> *Pantodrilus matsushimensis* lives in sand, wet by the sea.

<sup>h</sup> Weng (1946) claims to have demonstrated thermolabile and thermostable components.

<sup>i</sup> The location of luminous organs has been tabulated by G. Grimpe and H. Hoffman (*Tab. Biol.*, 6, 462-4, 1930).

<sup>j</sup> Red, white, and blue in *Lycoteuthis diadema*.

<sup>k</sup> Greenish yellow in *Spirula*.

<sup>l</sup> Bluish in *Oncaea*.

<sup>m</sup> According to a letter from Thomas E. Bowman regarding *Paraprionoe*.

<sup>n</sup> It has been claimed that the light comes from a fungus in some species.

<sup>o</sup> See McLeroy and Harvey (1951).

<sup>p</sup> Intense illumination of the luminous organ inhibits luminescence by affecting nerve stimulation.

<sup>q</sup> The purified luciferin is blue fluorescent in acid, yellow green in alkali (Strehler and McElroy, 1949).

<sup>r</sup> The location of luminous organs has been tabulated by G. Grimpe (*Tab. Biol.*, 6, 499-501, 1930).

<sup>s</sup> Report of Crozier in *Pyrosoma* at Bermuda.

<sup>t</sup> According to Hickling (1925).

<sup>u</sup> No luminous bacteria could be demonstrated by Haneda (1952) in *Yarella* or *Polyipnus*.

<sup>v</sup> *Argyroplecus* photophores show no marked fluorescence (Harvey, 1926).

<sup>w</sup> *Kaloplocamus* has intracellular luminescence, according to Haneda.

## EXPLANATION OF TABLE I

The groups are arranged phylogenetically in order of increasing complexity. References to authors and original papers are not given, since the complete literature and additional details regarding light production will be found in the book, *Bioluminescence*, by E. N. Harvey (Academic Press, N. Y., 1952), containing a bibliography of 1800 papers. Papers not included in the book are listed at the end of this article.

*Column 1* records the habitat, whether marine (M), fresh water (F), or terrestrial (T).

*Column 2* indicates the type of light, whether due to self-luminosity with intracellular (I), or extracellular (E) light emission from secreted material, or to symbiotic luminous bacteria ( $B_s$ ) always found in the luminous organ, or to parasitic luminous bacteria ( $B_p$ ) which have infected the living animal. Saprophytic luminous bacteria will grow on any dead organisms, especially fish and squid, and some false reports of luminescence stem from this source (the fish, *Harpodon*). Luminous fungi will grow on wood and many other vegetable materials.

*Column 3.* Knowledge of the cytology of the photogenic cell or the histology of the light organ is indicated by a + if the structure is well known, by a - if unknown and by  $\pm$  if further study is desirable. Mutation has been investigated only in bacteria, genetics only among the fungi. Knowledge of chemistry and physics of light emission is best known among bacteria, ostracods, and fireflies (columns 4-9).

*Column 4.* The necessity of dissolved molecular oxygen for light production is indicated by +. A - means that light will appear in complete absence of oxygen ( $Pt$  and  $H_2$ , or in excess hydrosulfite). A blank in this column and in columns 4-9 indicates knowledge is lacking.

*Column 5.* The luciferin-luciferase reaction is positive (+) if light appears when a hot water extract of the luminous cells allowed to cool (luciferin) is mixed with a cold water extract of the luminous cells allowed to stand until the light disappears (luciferase). The luciferin of one species will give no light with the luciferase of another luminous organism unless the two are closely related, such as different species of fireflies or different species of ostracods. No light appears on mixing luciferin (or luciferase) with luciferase (or luciferin) of a firefly and an ostracod, or *Odontosyllis* and an ostracod, or *Systellaspis* and an ostracod.

*Column 6.* If a nonluminous water extract of the luminous tissue containing magnesium emits light when the sodium salt of adenosine triphosphate (ATP) is added, the ATP reaction is positive; if no light the reaction is negative. A + and a - sign indicate positive in some species, negative in others.

*Column 7.* Marked inhibition of luminescence of single-celled organisms or of luminescent extracts by sunlight are indicated by + and no marked inhibition by —. A + and a — indicate that some species show light inhibition, others not. DN indicates that there is a day-night rhythm of luminescence such that the intact living organism does not luminesce in the daytime even if kept in the dark.

*Column 8.* Many luminous organs are brightly fluorescent in near ultraviolet light or become so after functional activity. The color of the fluorescent light (usually near that of the bioluminescence) is indicated. A — sign means no marked fluorescence.

*Column 9.* The color of the bioluminescent light is recorded, but too much reliance must not be placed on color estimation by eye observation. The wavelength of maximum emission is also given where spectrophotometric curves are known.

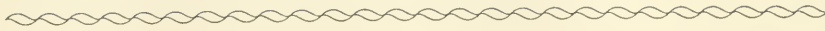


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# Luminescence Spectroscopy of Molecules and the Photosynthetic System\*

R. S. BECKER and M. KASHA  
Department of Chemistry, Florida State University,  
Tallahassee, Florida

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## I. Scope of Present Discussion

In recent years numerous advances in the interpretation of electronic states and transitions in complicated molecules have been made. We shall discuss the application of those results which we believe to be of importance for the chlorophylls in their role of energy transfer agents in photosynthesis. Our discussion will be qualitative and without the complications of spectroscopic nomenclature and symbolism. Moreover, we shall omit all discussion of such fine points concerned in the electronic transitions as symmetries, polarizations, vibrational fine structure, and assignments. We believe that these points, however intrinsically interesting they may be to the professional spectroscopist, will not be involved directly in the problem of photosynthesis.

On the other hand, we shall discuss all those spectroscopic aspects which we believe will be important in the capabilities for utilization and transfer of electronic excitation energy by the chlorophylls. As will be shown, this discussion will be limited perforce to the lowest electronic states of the molecules, namely, the ground (singlet) electronic state, the lowest triplet excited state, and the singlet excited states (our discussion requires the plural) involved in the red absorption band of the chlorophylls. Specifically, the Soret band and higher energy transitions we shall disregard, in accordance with the role of Internal Conversion discussed in Section II.

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The main points of our discussion will involve (1) the role of *n,π-transitions* in the chlorophylls, (2) the importance of *intercombinations* in these molecules, and (3) the possible interaction of the *ethylenic potential function* with the electronic transitions in the chlorophylls. Our interpretations will be applicable rigorously to the properties of the chlorophylls as physically isolated molecules *in vitro*. However, by discussing expected changes in properties of the molecules upon change in environment, we shall be able to extrapolate occasionally to the behavior of the molecule *in vivo* as a photosynthetic agent.

Omitted from our discussion will be any discussion of chemiluminescences, of the mode of energy transfer involving chlorophylls, and of other problems which are involved mainly in the biological system, such as accounting for the difference in the chlorophyll absorption bands in the living plant and *in vitro*.

## II. Importance of Lowest Excited Electronic States in Utilizability of Excitation Energy

### (1) Internal Conversion

Extensive studies in the field of molecular luminescence have revealed that only the lowest electronic state of a given series of electronic states in a molecule is capable of re-emitting its excitation energy. Upper electronic states of the series, upon excitation, lose energy thermally (by collisions) and radiationlessly go over into the lowest electronic excited state of the series. By series of states is meant states of one electronic multiplicity, or mode of electronic pairing (see Section V). This phenomenon of radiationless combination of excited states is known generally in the field of spectroscopy as *internal conversion* (for additional discussion, see Kasha, 1950). Its simplest consequence is that only the lowest excited singlet state is important in any energy transfer or energy utilization process: the higher excited states decay far more rapidly and generally cannot be expected to participate in any photochemical process.

### (2) Observation of Fluorescence

In unsaturated molecules, including the chlorophylls, the *π-electrons* give rise to most of the low-lying states corresponding to absorption

bands in the visible and ultraviolet regions (cf. Coulson, 1947, and Platt, 1951, for general discussions). A general study of luminescence properties of molecules (Kasha, 1950) shows that if the  $\pi$ -electronic states are the lowest singlet states of an excited series, fluorescence generally will be observed. If, however, the lowest excited singlet level is of an  $n,\pi$  type (cf. Section IV), the molecule will be generally a nonfluorescent one. In Section IV we shall interpret the behavior of chlorophyll fluorescence activation on this basis.

The intrinsic lifetime of the lowest singlet excited state will limit the probability of utilization of the excitation energy of that state. If the state is very short-lived, it may be that no energy transfer or photochemical process will be rapid enough to compete with spontaneous fluorescence emission. If a molecule has a high intrinsic quantum yield of fluorescence (or phosphorescence, see below) in an undisturbed (isolated) system, this property indicates excitation energy *availability*. However, if strong fluorescence actually is observed for the molecule in any reacting system, the lifetimes of fluorescence must be shorter than those for any other process and the observed quantum yield of fluorescence then indicates energy wasted. In other words, the spontaneous emission rate in such a case was comparable to the rates for some other energy utilization process. A few quantitative relations governing lifetimes and quantum yields will be given in the next section.

### (3) Lowest Triplet State of the Molecule

All normal molecules studied thus far, barring understandable exceptions, have exhibited lowest triplet excited states (Lewis and Kasha, 1944; Kasha, 1947). By normal molecules is meant here that an even number of electrons is present in the molecule, with electron pairing, so that diamagnetic, singlet ground electronic states result. The common electronic excitation is that in which the electron pairing is maintained: this gives rise to all the commonly observed absorption bands and the fluorescence phenomenon. Such transitions are labeled singlet  $\rightarrow$  singlet.

On the other hand, if electron pair uncoupling takes place, states of higher multiplicity are observed, the common state of this type being a triplet state in normal molecules, the singlet-triplet transition being designated an intercombination. Of course, many such triplet



states are possible for the molecule. However, in accordance with the above discussion of internal conversion, only the lowest triplet state will be of any importance in photochemical and other energy utilization processes.

We shall be interested in what role the lowest triplet state may play in the photosynthetic reaction. Outstanding in its importance in photochemical and energy transfer mechanisms is the long lifetime of the triplet state. In general, a triplet state will be longer lived by a factor of one million over the lifetime of a corresponding singlet state. This places the range of *intrinsic* triplet state lifetimes between  $10^{-4}$  second and 1 second for "normal" cases. There are several mechanisms by which intrinsic triplet state lifetimes may be varied considerably without quenching of excitation energy. These phenomena will be discussed mainly in Sections V and VI. It is likely, however, that if a triplet state of a chlorophyll may be excited easily, it may play an important part in any energy utilization process, on the basis of lifetime alone.

It is now generally recognized among spectroscopists that the lowest triplet state can be detected readily in most molecules by the study of low-temperature phosphorescence spectrum (Lewis and Kasha, 1944; Kasha, 1947; Kasha, 1950). The intrinsically long lifetime of phosphorescence generally makes its observation in fluid systems impossible, since in such cases the quantum yield and lifetime are reduced to immeasurable limits by the competitive collisional deactivations. Consequently, the phosphorescence emission of a molecule is always sought in rigid glass solvents. The high viscosity in such systems allows the long-lived phosphorescence to be observed. At lower viscosities, quenching will occur, and any phosphorescence emission observed will have a shorter lifetime and a diminished quantum yield compared with the intrinsic values.

In Section V we shall discuss the expected wavelength range and other characteristics of the lowest triplet states of the chlorophylls.

### III. Basic Quantitative Relationships

#### (1) Intrinsic Lifetime of an Excited State

In the previous section we have argued that an important criterion of utilizability of excitation energy is the intrinsic lifetime of the excited



state. In lieu of direct measurement of decay time constant (which in the absence of any form of quenching would of course be the intrinsic lifetime), we may make recourse to the classical relation (for discussion, see Lewis and Kasha, 1945; Kasha, 1950):

$$\tau^\circ = \left( \frac{1}{8\pi c \bar{\nu}_a^2 n^2} \right) (g_u/g_l) \left( 1 / \int_{\bar{\nu}_a}^{\bar{\nu}_2} \epsilon d\bar{\nu} \right)$$

This expression allows the intrinsic lifetime  $\tau^\circ$  of a luminescence to be calculated from the absorption band integral,  $\int \epsilon d\bar{\nu}$ , evaluated graphically. The average frequency ( $\text{cm}^{-1}$ ) of the transition  $\bar{\nu}_a$ , the refractive index of the medium  $n$  at the same frequency, and the multiplicity ratio  $g_u/g_l$  for the *upper* and *lower* states are the other variables. The constants are  $\pi$ , and  $c$ , the velocity of light.

## (2) Summation of Intrinsic Quantum Yields for a Molecule

We define quantum yield or quantum efficiency of a luminescence by

$$\Phi = \frac{\text{Number of quanta emitted}}{\text{Number of quanta absorbed}}$$

The total intrinsic quantum yield for a molecule (in the absence of external quenching) may then be described by

$$\sum_i \Phi_i = \Phi_F^\circ + \Phi_p^\circ + \Phi_{\text{int}} = 1$$

where  $\Phi_F^\circ$  is the intrinsic quantum yield of fluorescence,  $\Phi_p^\circ$  is the intrinsic quantum yield of phosphorescence, and  $\Phi_{\text{int}}$  is the intrinsic quantum yield for *internal degradation* by thermal steps. In the older literature the incorrect expression  $\Phi_F^\circ + \Phi_{\text{int}} = 1$  is assumed, leading to the conclusion that if no fluorescence is observed, internal degradation must predominate. Actually, in general the sum of  $\Phi_F^\circ + \Phi_p^\circ$  may approach unity (Kasha, 1950), so that  $\Phi_{\text{int}}$  may be negligible in many molecules, at least in rigid solvents.

It is of the utmost importance to understand that  $\Phi_F^\circ$  and  $\Phi_p^\circ$  are complementary in magnitude. Thus, if  $\Phi_{\text{int}} = 0$ , as seems to be the case in general for rigid molecules, then if  $\Phi_F^\circ = 0.2$ ,  $\Phi_p^\circ$  must equal 0.8. In other words, in a fluid solution, even if phosphorescence is *not* observed, due to collisional deactivation, the corresponding triplet state is excited with a probability of 0.8 for each absorbed quantum (and this is followed by deactivation, if fluid). Then, if this triplet state is in-

volved in a photochemical process, the limiting quantum yield of the photochemical reaction would be 0.8. Conversely, if, say, an energy transfer or photochemical reaction proceeds uniquely via the lowest singlet excited state, and if  $\Phi_F^\circ = 0.2$ , then the limiting value of  $\Phi_{\text{photochem}}$  will be 0.2.

### (3) Quenching: Lifetime and Quantum Yield

If we define observed lifetime of an excited state by  $\tau$ , and intrinsic lifetime by  $\tau^\circ$ , then for collisional bimolecular quenching

$$\frac{\tau}{\tau^\circ} = \frac{\Phi}{\Phi^\circ}$$

where  $\Phi$  is the *observed* quantum efficiency of a luminescence in the presence of partial quenching, and  $\Phi^\circ$  is the intrinsic quantum yield of fluorescence (*or* phosphorescence) as before.

Consequently, for collisional bimolecular quenching

$$\tau = \left( \frac{\Phi}{\Phi^\circ} \right) \tau^\circ$$

For example, if  $\Phi^\circ = 0.2$ , and  $\Phi = 0.02$ , then the observed lifetime will be 0.1 of the intrinsic lifetime of an excited state. In the older literature, the incorrect expression  $\tau = \Phi \tau^\circ$  was used; for the example given, the observed lifetime would appear to be 0.02 of the intrinsic lifetime. This error can be traced to the neglect of the complementary luminescence to the one observed, as the reader can confirm by the study of the expression given in Section (2). Obviously, the incorrect expression sets  $\Phi^\circ = 1$  for any luminescence.

## IV. Role of $n, \pi$ -Transitions in Spectra of Chlorophyll *a* and Chlorophyll *b*

First we shall give a rough qualitative description of  $n, \pi$ -transitions. Such transitions have been described by McMurry and Mulliken (1940), and more generally by Kasha (1950) and Platt (1953).

The designation  $n$  means nonbonding electron orbital. In addition to pure  $\pi$ -electron excitations, it is found that nonbonding electrons (referred to by chemists as "lone pairs") may be excited to unfilled  $\pi$ -electron orbitals in the molecule.

Examples of  $n \rightarrow \pi$  transitions are: (a) the excitation of a nonbonding  $2p$  orbital electron of the oxygen in formaldehyde  $\text{H}_2\text{C} = \ddot{\text{O}}$ : to

an excited (antibonding; cf. Coulson, 1947)  $\pi$ -orbital; (b) the excitation of a nonbonding electron of the N-atom in pyridine to an excited (antibonding)  $\pi$ -orbital.

For our purposes the characteristics of  $n \rightarrow \pi$ -transitions which are of interest are:

(1)  $n \rightarrow \pi$ -absorptions (even if allowed by spectroscopic selection rules) are weaker than corresponding  $\pi \rightarrow \pi$ -absorptions (Kasha, 1950). This means, in view of the relation discussed in Section III (1), that  $n, \pi$ -excited states will be much longer lived than analogous  $\pi, \pi$ -excited states, with the consequences discussed previously.

(2)  $n \rightarrow \pi$ -absorptions show a *blue shift* upon change of solvent from hydrocarbon type to hydroxylic type (Kasha, 1950; McConnell, 1952). Blue shifts of as much as a few hundred to a few thousand wave numbers ( $\text{cm}^{-1}$ ) have been observed. Recently it has been established (Brealey and Kasha, 1954) that these blue shifts may be ascribed largely to hydrogen bonding of the solvent to the  $n$ -electrons of the solute molecule.

(3) If  $n \rightarrow \pi$ -absorptions fall at lower energy or longer wavelength than any  $\pi \rightarrow \pi$ -absorption, then the molecule will be nonfluorescent (Kasha, 1950). Examples are many-fold, in which the nonfluorescence may be attributed to this juxtaposition of energy levels. Thus, all aliphatic ketones and many aromatic ketones and aldehydes are nonfluorescent and have  $n, \pi$  levels lower than  $\pi, \pi$ . In these,  $\Phi_F^\circ = 0$ , and  $\Phi_p^\circ \cong 1$ , so that (only) very strong phosphorescences are generally observed in rigid glass solutions. Other examples are most nitro compounds, quinones, azo compounds, and simple N-heterocyclics.

The application of these interpretations to the chlorophylls may be made as follows. Both chlorophyll *a* and chlorophyll *b* contain carbonyl groups,  $\text{C}=\text{O}$ . Isolated carbonyl groups will have ultraviolet  $n \rightarrow \pi$ -absorptions, similar to that in acetone; these will not interest us. However,  $\text{C}=\text{O}$  groups conjugated with other parts of the molecule may have  $n \rightarrow \pi$ -absorption at quite long wavelengths. Moreover, the more highly conjugated the carbonyl group, the stronger will the  $n \rightarrow \pi$ -absorption be, although in all cases somewhat less strong than pure  $\pi$ -electron absorptions. Moreover, such  $n \rightarrow \pi$ -absorptions should blue shift in hydroxylic solvents, or any solvents capable of forming molecular complexes specifically involving the  $n$ -electrons of the chlorophyll carbonyl groups.

The study of fluorescence activation of the chlorophylls by Livingston, Watson, and McArdle (1949) can be interpreted spectroscopically in terms of the above discussion. These workers found that chlorophyll *a* and chlorophyll *b* in hydrocarbon solvents showed very little fluorescence. However, especially in hydroxylic solvents such as alcohols and water, strong fluorescence was observed.

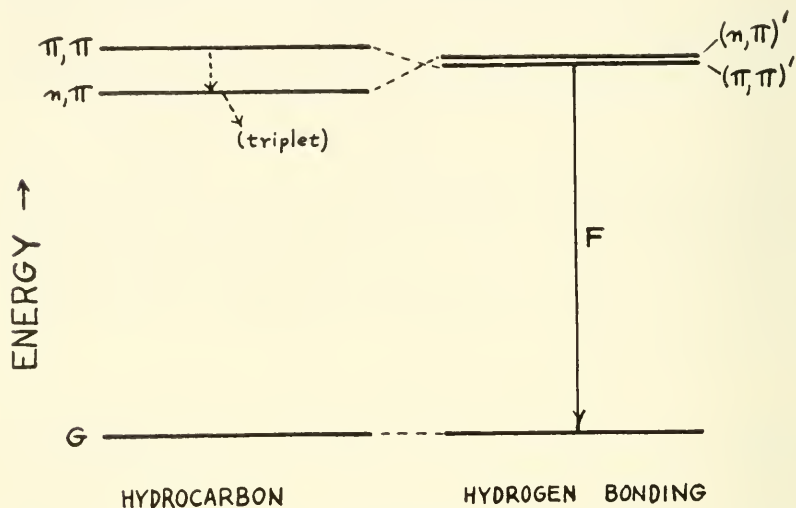


FIG. 1. Effect of hydrocarbon and hydrogen-bonding solvents on the frequencies of electronic transitions of a molecule with  $\pi, \pi$  and  $n, \pi$  levels close together. Ground states are equalized arbitrarily, and excited vibrational and triplet electronic levels are omitted.

Moreover, the lowest frequency absorption band of these two chlorophylls was more complex in hydrocarbon solvents (cf. their Figs. 4 and 5), since it is lower in apparent intensity in the latter (though not necessarily in integrated absorption) and, notably, shows evidence of a shoulder on the long-wavelength side of the main peak.

These results may be interpreted according to the diagram shown in Fig. 1. On the left side of the diagram we indicate the energy relationships which we picture for chlorophyll *a*. We assume that the slight shoulder shown in Livingston *et al.* (1949) is an indication of an  $n, \pi$ -transition just slightly lower in energy than the stronger,  $\pi, \pi$ -transition. Upon changing to a hydrogen-bonding solvent, the latter transition would be expected to undergo a normal red shift,

while the former undergoes a strong blue shift. Thus, we accept the discussion by Livingston *et al.* (1949) concerning the stabilization of a keto form by hydrogen-bonding solvents. However, we add to this the spectroscopic point that only the *hydrogen-bonded keto form* would be capable of fluorescence, if our energy scheme is correct.

For chlorophyll *b*, we observe that an additional conjugated carbonyl group is present (the formyl group). With two  $n,\pi$ -transitions possible for the *b* species, we anticipate a larger net  $\pi,\pi$ - $n,\pi$ -separation. In Fig. 4 of the paper by Livingston *et al.* (1949), a considerable shoulder on the long-wavelength side of the lowest absorption band for hydrocarbon solutions of chlorophyll *b* is found, which is absent for chlorophyll *b* in hydrogen-bonding solvents. We attribute the disappearance of this shoulder to a blue shift of the additional  $n,\pi$ -transition which we ascribed to the formyl group, as well as the blue shift of the  $n,\pi$ -transition, which is also present in chlorophyll *a*.

The above interpretation thus is capable of accounting for the differences between chlorophyll *a* and chlorophyll *b* spectra observed by Livingston *et al.* (1949), as well as for the appearance of fluorescence in the hydrogen-bonded molecules. Nevertheless, we note that the results of the Russian workers (Evstigneev, Gavrilova, and Krasnovski, 1950) emphasize the role of magnesium in the activation phenomena. The reconciliation of their results with our understanding of the spectroscopic aspects of this problem we have not yet achieved. However, we can state that we can picture no mechanism based on the hydration of the magnesium which would account for both spectroscopic observations, i.e., the activation of the fluorescence as well as the observed changes in the absorption spectrum.

Platt (1951) noted the possibility which we have discussed above, but he did not carry out the interpretation properly; in particular his idea of a negligible blue shift is not supported by subsequent work.

### V. Lowest Triplet State of Chlorophyll

We have in progress a comprehensive study of the luminescence emission properties of porphyrin-like molecules including chlorophyll *a* and chlorophyll *b*. In the following discussion we shall outline the theory and approach which we are using as a guide to this problem and shall indicate the positive results obtained thus far. At the outset

we can state that we have obtained a triplet  $\rightarrow$  singlet emission for chlorophyll *b*, a phosphorescence observed previously by other workers under uncertain conditions. We shall give a thorough analysis of the significance of this finding.

### (1) Singlet-Triplet Split: Expected Lowest Triplet Energy

The energy difference between the lowest excited singlet state ( $S'$ ) and the lowest triplet state ( $T$ ) (cf. Fig. 2) is called the *singlet-triplet split*<sup>\*</sup> in a molecule. This split is very variable (Kasha, 1947) from

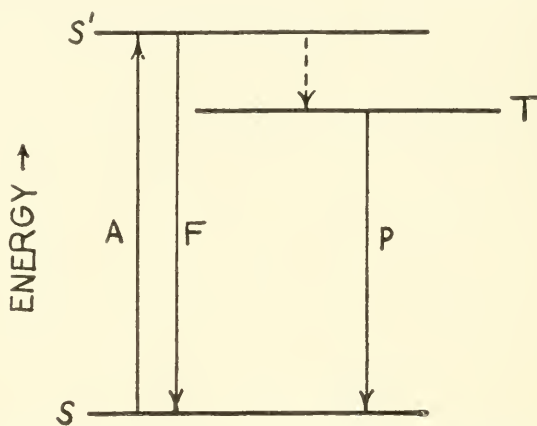


FIG. 2. Electronic levels important in energy utilization processes in a molecule (schematic). Only  $\pi, \pi$  levels are shown, and excited vibrational levels are omitted.

molecule to molecule. For example, in molecules like naphthalene and anthracene the split is very large, of the order of 10,000 to 12,000  $\text{cm}^{-1}$ . On the other hand, in dyelike molecules, such as the cyanines and many others, the singlet-triplet split is of the order of 2000  $\text{cm}^{-1}$ . If we classify a linearly or cyclically conjugated molecule like porphyrin as dyelike, then we might expect the singlet-triplet split to be of the order of 2000  $\text{cm}^{-1} \pm 1000 \text{ cm}^{-1}$ . In zinc tetraphenylporphyrin Dorough *et al.* (1951) have observed a singlet-triplet split of approximately 2500  $\text{cm}^{-1}$  (cf. their Fig. 11). In recent experiments in our

<sup>\*</sup> This definition assumes that the singlet and triplet states correspond in orbital configuration. If they do not, the energy difference given is called the *singlet-triplet separation*.



laboratory we have found an  $S'-T$  split of this same order of magnitude in metal phthalocyanines. We would expect the chlorophyll *a* and chlorophyll *b* molecules also to exhibit a similar split. Since the lowest frequency absorption of alcoholic solutions of these chlorophylls is at approximately 6500 to 6600 Å, and the fluorescences at approximately 6700 to 6800 Å, the phosphorescences or triplet-singlet emissions should occur at *approximately*  $2500\text{ cm}^{-1}$  lower in frequency, which would be at about 8050 to 8200 Å.

Calvin and Dorough (1947) originally reported a phosphorescence in a pure mixture of chlorophylls commencing at about 8000 Å and extending farther into the infrared. Later work by Calvin and Dorough (1948) on chromatographically separated chlorophyll *a* and chlorophyll *b* showed no phosphorescence in chlorophyll *a* and only a weak phosphorescence in chlorophyll *b*, commencing at 8600 Å and proceeding farther into the infrared. Further investigation by Livingston (1949) failed to reveal any emission from either chlorophyll *a* or chlorophyll *b*. Livingston believes that if a phosphorescence of chlorophyll exists, it is at longer wavelengths than 9000 Å or has a lifetime shorter than 0.01 second. It is obviously important that further work clarify these contradictions of experiment.

In our laboratory we have made preliminary observations on the phosphorescence of chlorophyll *a* and chlorophyll *b*. In agreement with Calvin and Dorough (1948), we found *no* phosphorescence for chlorophyll *a*. Furthermore, in agreement with Calvin and Dorough (1948), we did get a very good photograph of the phosphorescence of chlorophyll *b*. A small amount of chlorophyll *a* was present with the *b*-isomer; however, it is known that energy transfer will not take place in dilute solutions as rigid glasses at low temperature. Our exposure was sufficient to reveal one medium strong narrow band at 8650 Å. We believe there is no reasonable doubt as to the reality of this emission as a chlorophyll *b* phosphorescence. However, as discussion below will reveal, we do not simply relate this state with the photosynthetic step, contrary to the Calvin and Dorough (1947) hypothesis.

The experimental conditions under which our observations were made were as follows. We used a high-speed phosphoroscope having a resolving time of  $3 \times 10^{-4}$  second. The chlorophylls were dissolved in a rigid glass solvent formed by supercooling the dilute solution

(approximately  $10^{-7} M$ ) of the chlorophylls in standard EPA solvent mixture (cf. Kasha, 1947) (2 parts ethyl alcohol, 5 parts isopentane, 5 parts ethyl ether, measured by volume), at a temperature of  $77^\circ K$ . The chlorophylls were chromatographed on a column of packed sugar containing 3% starch, the bands developed by a mixture of 15% (volume) ethyl ether in petroleum ether. The absorption spectrum of chlorophyll *a* was spectroscopically free of pheophytin, chlorophyll *b*, and other plant pigments. The absorption spectrum of the chlorophyll *b* used indicated approximately 10–15% of chlorophyll *a* but no other plant pigments. We used ammonia-hypersensitized 1-N Eastman Kodak spectroscopic plates. The spectrograph used was a Hilger medium glass, Type E 495.

We are extending our observations to obtain complete vibrational structure of the phosphorescence emission and to obtain a measure of the quantum efficiency and lifetime (decay constant) of the emission. Our exposure time of several hours at a wide slit (1 mm) would seem to indicate that  $\Phi_p^\circ$  is rather small. However, we must point out that until the mean lifetime has been measured, we would have no comparison with the phosphoroscope resolution time quoted above. Only when the mean lifetime is known will it be possible to state with certainty whether our observation was made near the beginning of the phosphorescent decay, or near the tail end of the decay for each excitation cycle.

## (2) Effect of Electric and Magnetic Fields: Heavy Atom and Paramagnetic Atom Effects in Phosphorescence

Spectroscopically the mechanism of singlet-triplet transitions is understood in terms of a phenomenon known as spin-orbit interaction. For our purposes we can state that in all molecules there will be a spin-orbit interaction due to the electric fields of the nuclei of the atoms present in the molecule. This spin-orbit interaction may be strongly enhanced by the introduction of high atomic number or paramagnetic atoms into the molecule (cf. Kasha, 1950, and references therein). The effect of such introduction results in the following changes (1) the fluorescence quantum yield  $\Phi_F^\circ$  for the substituted molecule is lowered, (2) conversely,  $\Phi_p^\circ$  is increased greatly, and (3) the lifetime of the phosphorescence is decreased greatly. Spin-orbit

interaction is very sensitive to such effects, because of the high-power dependence of the probability of singlet-triplet transition on atomic number ( $Z^8$ ).

Applied to the porphyrin-like molecules, this means that replacement of magnesium ( $Z = 12$ ) by zinc ( $Z = 30$ ) should result in a pronounced enhancement of the phosphorescence and a considerable shortening of its mean lifetime. Replacement of magnesium by copper ( $Z = 29$ ) should have an additional strong effect due to the magnetic field produced by the unpaired copper  $d$ -electrons. Thus, Calvin and Dorough (1947, 1948) found such effects in zinc and copper chlorin and porphyrin derivatives. It is in fact an old observation in the porphyrin fluorescence field that the porphyrin derivatives of paramagnetic metals such as iron and copper, are nonfluorescent. We expect to find these strongly phosphorescent.

In our laboratory we have made some preliminary investigations on magnesium and copper phthalocyanines. The magnesium phthalocyanine is strongly red fluorescent, while the copper phthalocyanine shows no fluorescence. However, copper phthalocyanine shows a strong infrared phosphorescence at  $77^\circ \text{K}$ , in conformity with expectation.

A different sort of magnetic or electrical effect is also possible for heavy or paramagnetic atoms *in the neighborhood* of the excited molecule. It has been demonstrated (Kasha, 1952) that heavy-atom fluorescence quenchers induce the conversion of excited singlet energy to triplet energy. It is undoubtedly true that similar quenching effects by paramagnetic molecules such as  $\text{O}_2$  would be due to the same mechanism. It may turn out that these field effects will have some influence on the energy processes in chlorophylls in the photosynthetic system.

### (3) Nature of Lowest Triplet State of Chlorophyll

According to our discussion in Section IV, both  $\pi, \pi$  and  $n, \pi$  excited singlet states may be present in the region of the red absorption band of chlorophyll *a* and chlorophyll *b*. If this is so, and that can be established rigorously by further experiment, we would be compelled to consider that *below* the lowest excited singlet state there will be at least two triplet states in both chlorophyll *a* and chlorophyll *b*, one

triplet of an  $n,\pi$  type, and one triplet of a  $\pi,\pi$  type. In addition, in chlorophyll *b*, an additional triplet of an  $n,\pi$  type may result from an  $n$ -electron of the formyl group in its triplet excitation to a  $\pi$ -molecular orbital.

This discussion may appear rather complicated, but fortunately the questions raised are accessible to experimental investigation.

#### (4) Role of Lowest Triplet States of Chlorophylls in Energy Transfer

In all the published discussions of excitation energy transfer involving chlorophylls, the main emphasis has been on the study of fluorescence. It is obvious that if  $\Phi_F^\circ$  is of the order of magnitude of 0.1, the use of fluorescence as a criterion for energy transfer focuses attention on the minor part of the possible available energy. That the remaining 90% of the absorbed energy must be accounted for to explain the efficiency of observed photosynthesis in plants has been indicated previously, e.g., by Livingston (1949).

When the quantum efficiency of chlorophyll phosphorescence has been determined, we shall be in a position to evaluate whether the lowest triplet state receives the missing 90% of the energy. If any considerable amount of the excitation energy actually reaches the lowest triplet state, then it may be entirely possible for this state to be important in the energy transfer processes (preceding the photosynthetic primary step). In fact, it is entirely possible that chlorophyll *a* may transfer its excitation energy to chlorophyll *b* as far as *triplet* state energy is concerned. Of course, this would be just the reverse of the commonly accepted course of energy transfer between chlorophylls, deduced from fluorescence studies. We emphasize that we merely wish to point out the possibility of such a transfer, and not to predict its necessity.

We would be inclined to favor the idea of net energy transfer from chlorophyll *a* to chlorophyll *b* on two points: first, chlorophyll *b* is the minor component of chlorophylls in many living plants. It thus would play a minor part in light absorption by the plant, and energy transfer from chlorophyll *b* to chlorophyll *a* would have a minor role in photosynthesis. On the other hand, the spectroscopic and structural distinctions between chlorophyll *a* and chlorophyll *b* (especially if the

*b*-isomer has a readily available triplet state) may make chlorophyll *b* the important intermediate acceptor of the excitation energy. Thus, a net transfer of energy from chlorophyll *a* to chlorophyll *b* may be important. Moreover, the fact that chlorophyll *a* is a major component of plant chlorophylls would make its dominant light absorption the chief role of this isomer in photosynthesis.

In the next section we shall discuss one additional spectroscopic phenomenon which may dominate the properties of the chlorophylls in energy utilization. This involves the electronic interaction of the vinyl group present in chlorophylls and the possibility that it behaves as an energy dissipator in isolated chlorophylls *in vitro*. This may account for possible low quantum yield of luminescence of chlorophyll as isolated molecules.

## VI. Interaction of Low Excited States of Chlorophyll with Torsional Potential

The theory of the electronic-vibrational interactions in the ethylene molecule has been developed by Mulliken (1933) and Mulliken and Roothaan (1947), with particular reference to the effect of twisting or torsion about the double bond. This type of distortion is unique, among possible intramolecular distortions, in *lowering* vastly the energy of the *excited* singlet and triplet states (cf. Fig. 3) of ethylene.

The effects of this torsional potential as it is called are profound in determining the spectroscopic behavior of the molecule. For example, the absorption spectra change shape upon change of temperature and viscosity, and no fluorescence is observed (Potts, 1954). The reasons for this are clear from the diagram. Absorption is mainly to a molecule distorted *far* from its most stable configuration, quite the opposite of normal one-electron excitation in molecules. Upon cascading through torsional levels under the influence of collisions, the molecule reaches a minimum from which no emission could be observed at accessible wavelengths. Even in methylethylenes in rigid glasses (Potts, 1954) no fluorescence can be observed. However, in *trans*-stilbene (*trans*-diphenylethylene), fluorescence can be observed in rigid glasses or crystals, though not in fluid solutions (Kasha, 1950). Such behavior is general in complex ethylenic molecules.

Apparently, the phenyl groups are sufficiently large to inhibit the



torsional mode of vibration, probably by introduction of a barrier (dotted curve, Fig. 3) on the potential curve (we disregard the similar barrier in the ground state; it introduces no new feature). Consequently, absorption is to a nearly equilibrium configuration, with re-emission of fluorescence directly observable.

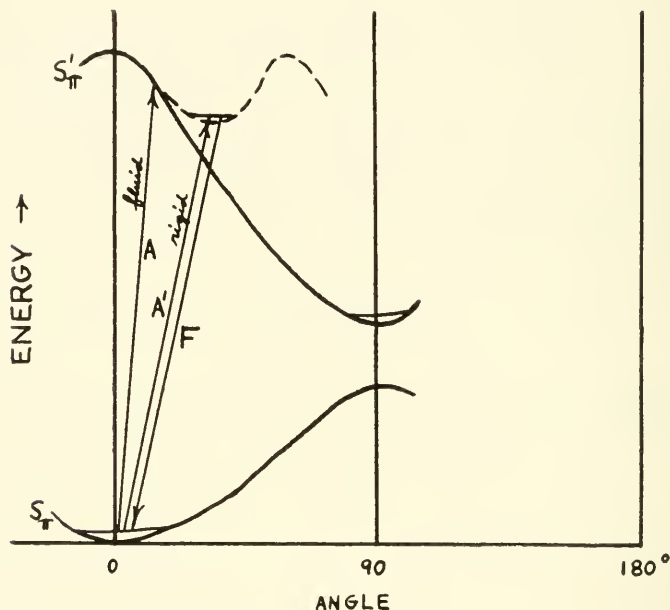


FIG. 3. The variation of potential energy with twist of the double bond in ethylenic molecules. The dotted curve represents a barrier to torsion introduced by inhibition of twisting motion in rigid solvents by substituted groups on the ethylene. *A*, fluid; *A'*, rigid.

The vinyl group  $-\text{CH}=\text{CH}_2$  in the chlorophylls is conjugated with the main  $\pi$ -electron system of the molecule. If this vinyl group is free to undergo torsion upon excitation, it is possible that in the isolated molecule this could serve as the chief dissipator of electronic excitation energy. Its efficiency as a dissipator would depend on the magnitude of the interaction of the main  $\pi$ -electron system with the torsional potential. The low quantum yield of fluorescence in alcohol solution in both chlorophyll *a* and chlorophyll *b* may be due to such an interaction. The absence of phosphorescence in chlorophyll *a* and



the possible low quantum yield of phosphorescence (not yet determined) of chlorophyll *b* would be similarly accounted for.

In the living cell, it is possible that these dissipative effects, if actual, would be absent. This could be the secret of chlorophyll in the living cell. Certainly, if electronic energy is dissipated as described above, and if attachment of chlorophyll to some other molecule is through the vinyl group *in vivo*, then most of the absorbed light energy could become photochemically available.

## VII. Conclusion

From our analysis of the spectroscopic interpretation of the chlorophyll problem, it is apparent how little experimental investigation has been made on the nature of the lower electronic states, which would be involved in energy utilization processes. As a result, much of our discussion has necessarily been speculative. However, we hope that the viewpoint presented will stimulate further examination of these points. In our developing research program we shall have the opportunity to investigate experimentally many of the interpretations made.

Since our discussion is a preliminary one, we have refrained from a discussion of the follow-steps in photosynthesis that have been proposed recently. Once the primary step of energy utilization for chemical reaction initiation has been established for the chlorophyll system, the relation to the follow-steps should become apparent. Our basic aim is to establish which electronic state of chlorophyll is involved in this primary energy step, as contrasted with simple light absorption.

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## DISCUSSION

**Dr. Duysens:** I should like to point out two things which seem to argue against the suggestion made by Mr. Becker that the light energy is passed on to the photosynthetic reaction via a triplet state of chlorophyll *b*.

The striking similarity between the action spectrum of the fluorescence of chlorophyll *a* and the action spectrum of photosynthesis of the many photosynthetic organisms investigated indicates that the energy absorbed by the various pigments is passed on to the fluorescent (lowest singlet) state of chlorophyll *a* and in this way becomes active in photosynthesis. It might now be argued that the energy is passed from chlorophyll *a* to the triplet state of chlorophyll *b*. As far as I know, no mechanism is known by which such a transfer will be accomplished with *high efficiency*.

There is further the fact that many algae do not contain chlorophyll *b*. The pigment common to all of them is chlorophyll *a*. Apparently, an energy transfer from *a* back to a triplet state of *b* is, in general, not necessary for photosynthesis. It seems therefore not necessary to postulate that such a transfer back to *b* occurs.

**Dr. Kasha and Mr. Becker:** The philosophy of our proposal concerning energy transfer between chlorophyll *a* and chlorophyll *b* has apparently not been made clear. Our aim was merely to point out a possibility that had been overlooked previously. Most of the conferees seem to have taken it for granted that we implied that energy transfer from chlorophyll *a* to chlorophyll *b* (via triplet states) was a *necessary* phenomenon, which was not our intention.

Thus, in answer to Dr. Duysens' second point, it is obvious that if algae do not contain chlorophyll *b*, energy transfer between chlorophyll *a* and *b* cannot be considered, in *either* direction. His statement is not a valid criticism of our suggestion on energy transfer.

In answer to Dr. Duysens' first point, it is true that there is no highly developed mathematical theory to account for a transfer of energy from the triplet state of one molecule to another. However, such a possibility is qualitatively very plausible and probably is quite a common photochemical mechanism. Highly efficient excitation of triplet states is common; the long lifetime of the latter makes a highly efficient transfer of energy plausible.



The main quantitative theory on energy transfer, due to Förster, predicts that efficiency of energy transfer is proportional to intensity of absorption of the acceptor molecule. This would argue against our suggestion. However, it is doubtful whether Förster would insist that his mechanism would apply to all types of energy transfer.

We should like to point out that Dr. Duysens' statement (that the similarity of the "action spectrum of fluorescence" of chlorophyll *a* and the "action spectrum of photosynthesis" requires that the "fluorescent" level be involved in photosynthesis) is incorrect. In the normal excitation of phosphorescence the "action spectrum" of fluorescence and phosphorescence are necessarily identical, since both first involve lowest singlet-singlet absorption.

**Dr. Mason:** Would you describe the operations which characterize fluorescence and phosphorescence? Have phenomena such as delayed fluorescence or premature phosphorescence been observed? What operations must be performed in order to decide in which category the emission falls?

**Dr. Kasha and Mr. Becker:** Dr. Mason's first question is answered in the published literature (Kasha, 1947; Lewis and Kasha, 1944).

In answer to the second question, there is no sharp distinction between fluorescence and phosphorescence of molecules on the basis of lifetime. Thus, fluorescence is *usually* very short lived, in the range  $10^{-8}$  to  $10^{-10}$  second (mean life) because the emission corresponds to intense absorption bands (cf. Section III, 1, our paper). However, singlet-singlet luminescence, or fluorescence may be long lived, as long as  $10^{-5}$  second ("delayed" has unhappy connotations) if the singlet-singlet absorption is weak. On the other hand, phosphorescence lifetimes are *usually* long, in the range 10 to  $10^{-4}$  second. Nevertheless, under the action of certain perturbations, such as substitutions in the molecule involving atoms of high atomic number (e.g., Br I), the inherent phosphorescent lifetime may be shortened as much as by a factor of 1000. Thus, a  $10^{-7}$ -second phosphorescence could be observed. Obviously, there is overlap between lifetimes of fluorescence and phosphorescence.

Distinctions which are qualitative may be made, however, between fluorescence and phosphorescence as follows: (1) The excited state giving rise to phosphorescence is paramagnetic. This has been proved by direct susceptibility measurements in one case of phosphorescence. It is a difficult measurement and cannot be applied easily enough to

make it worth while. There is a possibility that the new *paramagnetic resonance absorption* methods can be used to detect triplets generally. (2) The phosphorescence lifetime is sensitive to high-atomic-number-atom substitution in a unique way, whereas fluorescence lifetime is generally insensitive to such substitutions. However, in some simple molecules (e.g., acetone) this distinction is not very clear, since substitution has several effects. It is possible that the new method of environmental perturbations (Kasha, 1952) may resolve such difficulties. (3) The clearest distinction between fluorescence and phosphorescence is the wavelength of the emission, if *both* emissions exist. In a few molecules, only one or the other emissions exists; this is a rare circumstance, but where it occurs, recourse can be made to steps (1) or (2) above. However, when both emissions can be observed (for which, of course, rigid glass solutions at low temperatures are used; see text), it is always true that phosphorescence is the longer-wavelength emission, whereas fluorescence is the shorter-wavelength emission (see text on "singlet-triplet splits"). Moreover, it can also be said that, if two emissions are found for a molecule, the shorter-lived emission is fluorescence, and the longer-lived is phosphorescence, regardless of their absolute values. It is virtually inevitable, spectroscopically, that these values will be quite clearly separated in magnitude.

This third distinction will probably serve as the clearest and most valuable answer to Dr. Mason's questions.







## Light Saturation of Delayed Light Production in Green Plants\*

WILLIAM ARNOLD

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

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Three years ago it was shown in this laboratory (Strehler and Arnold, 1951) that green plants emitted light for some seconds after being illuminated. The action spectrum for the delayed light was shown to be the same as that for photosynthesis, and thus it was chlorophyll which absorbed the light energy that was reemitted. Later experiments have shown (Arnold and Davidson, 1954) that the emission spectrum of the delayed light is the same as the fluorescent spectrum of chlorophyll in the living plant, and thus that it is chlorophyll which emitted the delayed light.

Although photosynthesis and delayed light production seem to be closely connected, a fact that has been emphasized in the past, in the manner of light saturation they are very different. Figure 1 gives the two curves for continuous light made on aliquots of the same *Chlorella pyrenoidosa* suspension. As can be seen, the delayed light production saturates at an intensity very much lower than photosynthesis. In the hope of understanding this difference, experiments have been done with short flashes of light.

A *Chlorella* suspension is pumped from a darkened container through a glass tube, a length  $D$  cm of which is illuminated with intensity  $I$ . From the rate of pumping, the diameter of the tube, and  $D$ , the time  $T$  that each cell spent in the light can be calculated. After flowing in the dark for a short distance, the suspension passes in front

\* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

of a photomultiplier which measures the intensity  $S$  of the delayed light.

It is found that if  $T$  is smaller than a few hundredths of a second, then  $S$  is a function of the product  $I \cdot T$ , that is,  $S$  depends only on total energy  $E$  in the light flash.

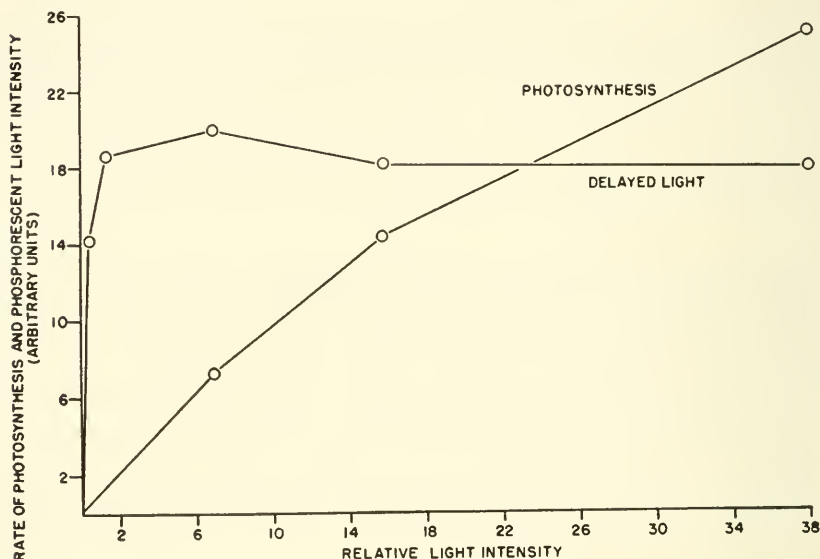


FIG. 1.

Figure 2 gives the result of the experiment. The insert gives the beginning of the curve for energies expressed as quanta per square centimeter for light of wavelength approximately 6500 Å.

Let us assume that the saturation shown by the curve is due to exhaustion of some substance  $K$ , and that the light changes  $K$  into  $KB$ . The return of  $KB$  to  $K$  generates the excited chlorophyll which emits the delayed light.

Let  $X$  = amount of  $K$  at any time

$X_0$  = amount of  $K$  at the beginning of flash

$\sigma$  = cross section for the reaction  $K \rightarrow KB$

$k$  = constant.

We further assume that  $S$ , the delayed light intensity at the end of the flash, is given by  $k$  times the amount of  $KB$ , that is, by

$$S = k(X_0 - X)$$

Since it is known that the reaction  $KB \rightarrow K$  is slow, as is shown by the decay curve of the delayed light, we have during the light flash,

$$\frac{dX}{dt} = -\sigma I X$$

This has the solution

$$X = X_0 e^{-\sigma I T} = X_0 e^{-\sigma E}$$

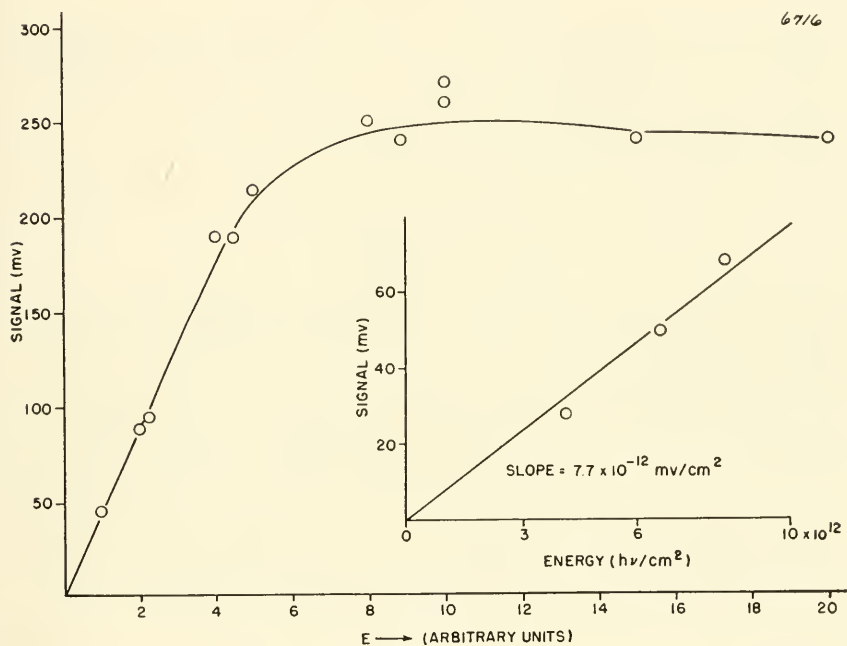


FIG. 2.

Therefore, the intensity of the delayed light will be given by

$$S = kX_0(1 - e^{-\sigma E})$$

The initial slope of the curve will be

$$\frac{dS}{dE} = kX_0\sigma$$

and the saturation value given by

$$S = kX_0$$

Therefore, the cross section for the reaction can be found by dividing the initial slope by the saturation value.

From the data given in Figure 2 it can be seen that

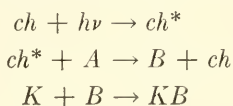
$$\sigma = \frac{7.7 \times 10^{-12}}{250} = 3.1 \times 10^{-14} \text{ cm}^2$$

Since the cross section for the absorption of light by chlorophyll in the living plant has been given (Arnold and Oppenheimer, 1950) as

$$0.5 - 1.4 \times 10^{-16} \text{ cm}^2$$

it can be seen that the reaction  $K \rightarrow KB$  has a cross section 200–600 times larger than a single chlorophyll molecule.

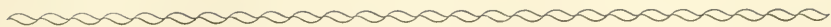
This large cross section, as well as the early saturation of the delayed light, can be understood in the following way. Let



be the reactions involved in the early steps of photosynthesis and delayed light production (here  $ch$  stands for chlorophyll and  $ch^*$  for excited chlorophyll). The large cross section is due to the ability of any  $B$  to combine with one of the  $K$ 's, since the ratio of chlorophyll to  $K$  is supposed to be very large and the reaction  $K \rightarrow KB$  is thought to be very fast. If it is now believed that either the amount of  $B$  or the amount of  $B$  plus  $KB$  determines the rate of photosynthesis, it is seen that the saturation of photosynthesis can take place at a much higher light intensity than the saturation of the delayed light.

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## Fluorescence Spectrophotometry of Photosynthetic Pigments

C. STACY FRENCH

Department of Plant Biology, Carnegie Institution of Washington,  
Stanford, California

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Absorption spectrophotometry has played a large part in the development of modern biochemistry. Fluorescence spectrophotometry, on the other hand, has not been used as widely because there are many more substances that can absorb light than can reemit fluorescent light; furthermore, the measurements are somewhat more difficult to make. However, in suitable cases the value of fluorescence spectrophotometry equals or exceeds that of absorption spectrophotometry for pigment identification in live cells and in extracts and for quantitative analysis. Also, it is ideally suited to the study of energy transfer between pigments.

During the course of an investigation on energy transfer between the pigments of red algae it became apparent that the basic data available on the fluorescence spectrophotometry of photosynthetic organisms and of their purified pigments is very meager. The spectrographic location of fluorescence peaks determined largely by Dheré has been reviewed by Rabinowitch (1951). Vermeulen, Wassink, and Reman (1937) measured the fluorescence energy distribution curves of *Chlorella*, the purple bacterium *Chromatium*, a plant extract containing chlorophyll *a* and *b* and an extract of purple bacteria containing bacteriochlorophyll. Zscheile and Harris (1943) made precise measurements of the fluorescence spectra of pure chlorophylls *a* and *b* in various solvents. Van Norman, French, and Macdowall (1948) measured the fluorescence curves of two red marine algae and of a water extract from one of them. Duysens (1951) published the

fluorescence spectrum of the red alga *Porphyra lacineata* in a study of energy transfer between photosynthetic pigments. This study was extended to pigments of blue-green algae, purple bacteria and *Chlorella* (Duysens, 1952). French and Young (1952) also measured fluorescence spectral energy distribution curves of photosynthetic pigments in live cells and in extracts. They have reviewed the work prior to 1951 on absorption, action, and fluorescence spectroscopy of photosynthetic pigments (Hollaender, Ed.).

Although the experiments here described have been limited to the study of photosynthetic pigments, the possible uses of these methods in other fields of biology and biochemistry, particularly in the field of luminescence, are evident. This paper will describe briefly the apparatus constructed for automatically plotting the spectral energy distribution of weakly emitting light sources such as fluorescing leaves and will illustrate the use of such measurements in studying the properties of pigments in solution and in living cells. Most of the material has been taken from more detailed papers already published or now in preparation; only a superficial survey will be attempted here. Some progress reports in this field have been published (Carnegie Institution of Washington Year Books, 1948-1953).

### Apparatus

Quantitative measurement of a fluorescence excitation spectrum requires illumination of the sample with bright light in a narrow spectral range and of known total energy as measured by a thermopile. This incident light is converted in the sample, usually with a very low efficiency, into fluorescent light that is emitted in all directions. To measure the spectral energy distribution of the weak fluorescent light, as much as possible of it must be put through a monochromator and the intensity of its various wavelengths determined.

In order to make such measurements practical it is necessary to start with a bright light source. Our high intensity source is a Bol type of high-pressure mercury lamp made by the Huggins Laboratories in Menlo Park, California. This is similar to the GE H6, but it dissipates twice the power in a narrower capillary. This lamp gives a very high intensity continuous spectrum as well as the mercury lines which are very greatly broadened. Even in the red part of the spectrum this



source is brighter than a tungsten lamp. We run the lamp on alternating current and thereby get 120 light pulses per second. An image of the lamp is focused on the slit of a monochromator by a spherical mirror. The monochromator is usually set to isolate a total band width of  $10 \text{ m}\mu$ . Stray light is removed by filters. As shown in Fig. 1, a block diagram of the apparatus, this incident light may be measured by means of a thermopile in a sliding mount or may be allowed to fall upon the sample.

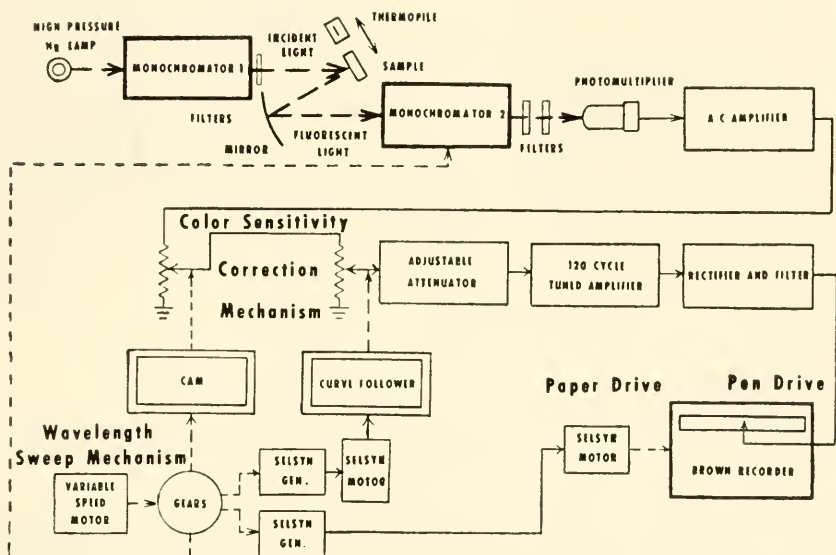


FIG. 1. Block diagram of the apparatus for recording fluorescence spectra.

Most of the fluorescent light given off by the sample escapes, but some of it is caught by another curved mirror and sent into an analyzing monochromator, which also isolates  $10 \text{ m}\mu$ , total band width. Behind this monochromator is a filter to remove reflected incident light and one to give some correction for the wavelength sensitivity of the photomultiplier tube. The electrical output of this photomultiplier tube is amplified and passed through an attenuator which is linked to the wavelength drive of the analyzing monochromator by a cam. The cam is cut to compensate for the variation of photomultiplier sensitivity with wavelength and also to include the varying

transmission of the monochromator with wavelength. The cam takes care of most of the corrections, but some residual errors are later removed by another attenuator drive by a photoelectric curve follower. The residual correction curve is put on a frosted lucite drum with a soft pencil and can easily be adjusted. The corrected electrical signal, now proportional to the intensity of the light emitted from the sample at each wavelength, is separated from random noise by

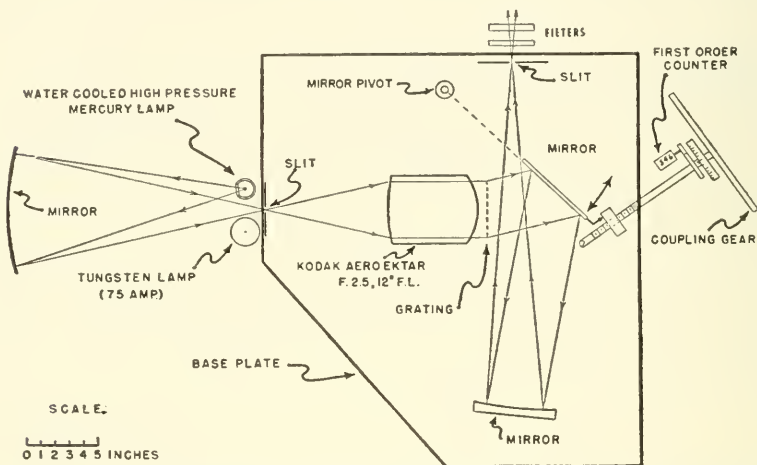


Fig. 2. Transmission grating monochromator used for producing the incident light.

means of a 120~tuned amplifier, rectified to direct current and used to drive the pen of a Brown recorder. The paper in the recorder is moved synchronously with the wavelength drive by means of a Selsyn motor. It is therefore possible to vary the speed of wavelength sweep through various parts of the spectrum during a run.

The monochromators are designed for high light gathering power and are built with approximately 10 by 10 cm gratings. The monochromator, Fig. 2, used to isolate the incident light has a replica grating with a blaze angle producing a high efficiency in blue. The wavelength setting of this instrument is read directly on a counter. Figure 3 shows the arrangement of the incident and the fluorescent beams as well as the calibrating lamp. The color sensitivity correction cam and the final correction curve are made with light from a stand-

ard lamp of known color temperature reflected off a magnesium oxide block in the sample holder and interrupted by a 120-cycle chopper. The apparatus retains its calibration for months.

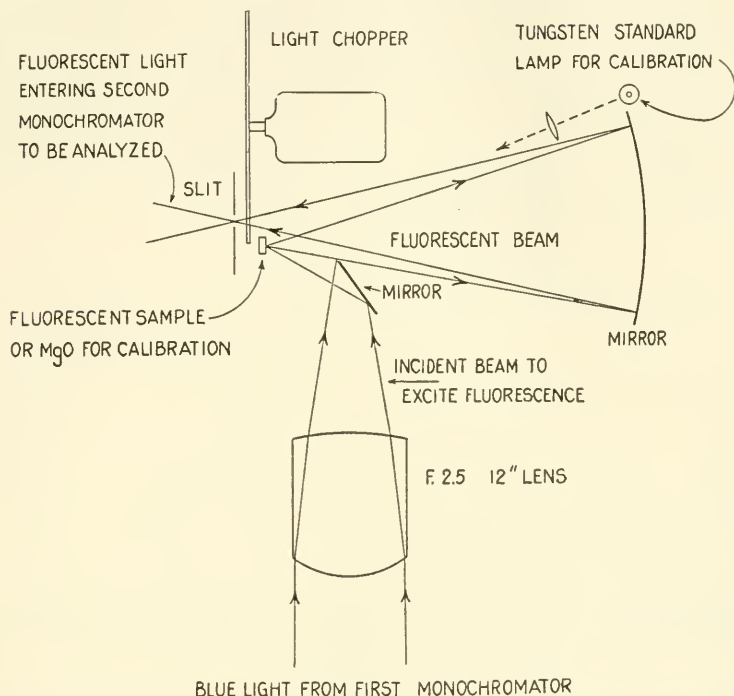


FIG. 3. Arrangement for illuminating the sample and for collecting the fluorescent light.

Figure 4 shows the monochromator used to separate the spectrum of the fluorescent light into its different wavelengths. This analyzing monochromator uses a reflection grating. The grating is rotated at an adjustable speed by a motor which is also coupled to the cam and the curve follower as illustrated in Fig. 1.

### Fluorescence Spectra of Extracted and Purified Pigments

#### Phycoerythrin

Figure 5 shows the absorption and fluorescence spectra of a sample of phycoerythrin. Comparison of the absorption and fluorescence

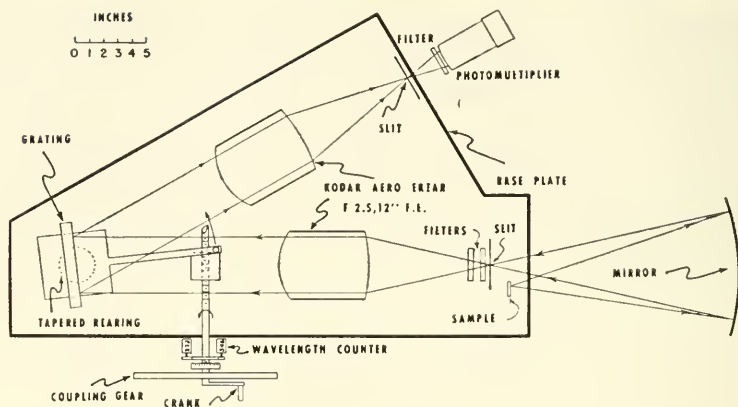


FIG. 4. Reflection grating monochromator used for analyzing the fluorescent light.

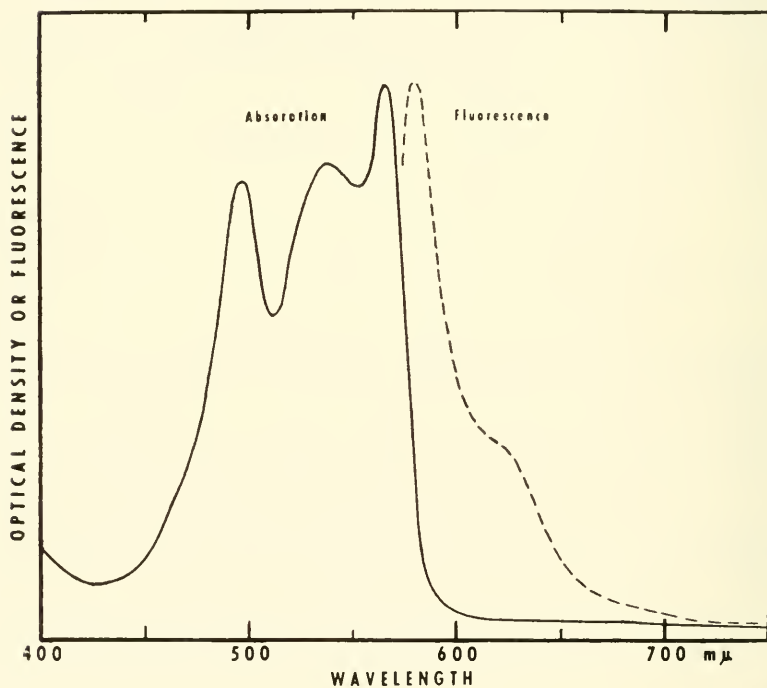


FIG. 5. Absorption and fluorescence spectra of pure phycoerythrin in water. Measurements of Dr. Violet K. Young on a sample of pure phycoerythrin kindly given us by Professor Lawrence R. Blinks. (Redrawn from Rabinowitch, 1951, Fig. 23.9A.)

curves of this pure pigment illustrates two well-known facts. First, the fluorescence spectrum looks somewhat like a mirror image of the absorption curve, at least two peaks in this case appear to be reflections of two of the absorption peaks. Second, the major fluorescence peak is universally located near an absorption peak, but at a longer wavelength. The situation that can cause a great deal of trouble in fluorescence spectroscopy is the overlapping of the absorption and the fluorescence bands. Here we see that the left-hand part of the fluorescence curve can be very strongly absorbed by the pigment itself

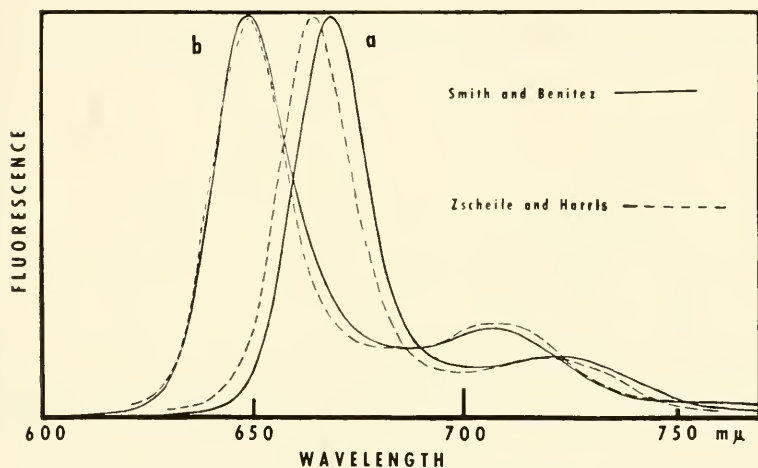


FIG. 6. Fluorescence spectra in ether of pure chlorophylls *a* and *b* of Smith and Benitez compared with the recalculated curves of Zscheile and Harris (1943).

whereas the right-hand part is outside of the absorption region of the pigment. This means that except in extremely dilute solutions the observed curve is very likely to be greatly distorted by internal reabsorption of the fluorescent light. This difficulty can be avoided, at least in solutions, by using very low concentrations or thin layers. Reabsorption within the sample can be the cause of a great deal of trouble in interpreting the spectra of living organisms which have a high pigment content.

#### Chlorophylls *a* and *b*

Since Dr. James H. C. Smith and Mr. Allen Benitez have recently

been redetermining the absorption constants of purified chlorophylls in this laboratory, we had an excellent opportunity to repeat the measurements of Zscheile and Harris on the fluorescence spectra of freshly purified chlorophylls *a* and *b* in ether. The new curves are compared in Fig. 6 with those of Zscheile and Harris which have been recalculated to correct for the variation of the effective slit width with wavelength due to their use of a prism. The need for such a correction of their curves was pointed out to us by Dr. L. N. M. Duysens. The agreement of the chlorophyll *b* curves is close, but the difference between the two chlorophyll *a* curves seems to be well outside the

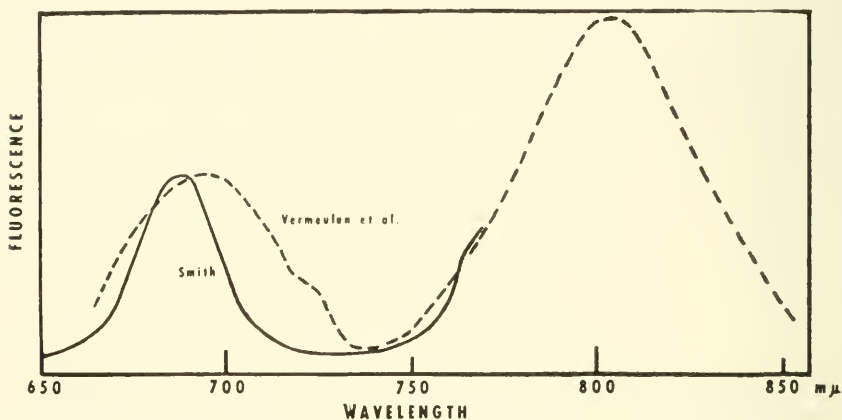


FIG. 7. Fluorescence spectrum in ether of "bacteriochlorophyll," measurements of Vermeulen, Wassink, and Reman (1937) and of this laboratory.

experimental error of either laboratory. Dr. Smith's absorption measurements of his chlorophyll *a* also show a somewhat similar wavelength shift. It appears that the chlorophyll *a* of the two laboratories may actually have been composed of different isomeric fractions.

### Bacteriochlorophyll

The dotted curve in Fig. 7 shows the fluorescence spectrum of bacteriochlorophyll published by Vermeulen, Wassink, and Reman (1937). The figure also shows the measurements of partially purified bacteriochlorophyll prepared by Dr. Smith and Mr. Benitez. Presumably the differences between the curves are due to the use of



narrower slit widths in this laboratory. We were rather surprised to confirm the finding of a fluorescence peak at  $687\text{ m}\mu$  since bacteriochlorophyll does not have a corresponding absorption band.

This bacteriochlorophyll preparation was separated chromatographically by Dr. Smith into three fractions—one blue, one green, and one

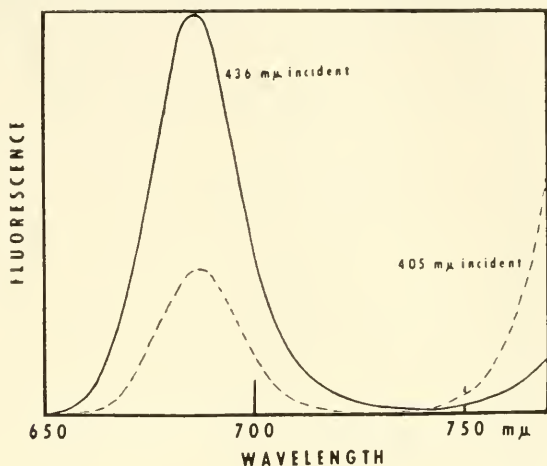


FIG. 8. Fluorescence spectrum of the blue fraction of a bacteriochlorophyll preparation in ether. (Smith, unpublished.)

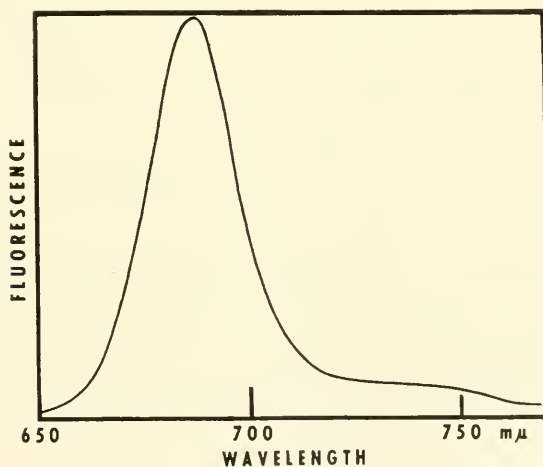


FIG. 9. Fluorescence spectrum in ether of the green pigment accompanying bacteriochlorophyll. (Smith, unpublished.)

pink. The fluorescence spectrum of the blue fraction when illuminated by two different incident wavelengths is shown in Fig. 8. Now it is ordinarily found that a pure substance illuminated with different wavelengths gives the same fluorescence spectrum, but this preparation gives different spectra for the two incident wavelengths. It is clear that the material fluorescing at  $687\text{ m}\mu$  absorbs at  $436\text{ m}\mu$  more strongly than it does at  $405\text{ m}\mu$ , since  $436\text{ m}\mu$  excites the  $687\text{-m}\mu$  band

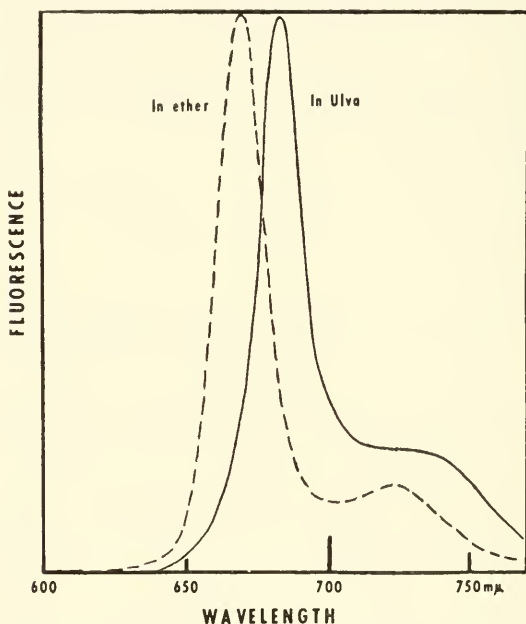


FIG. 10. Fluorescence spectrum of a thin layer of a live green plant as compared with an ether solution of chlorophyll *a*. (Smith, unpublished.)

more than it does the near infrared fluorescence, whereas  $405\text{-m}\mu$  incident light does the reverse. The green fraction, Fig. 9, of the bacteriochlorophyll preparation, however, shows only the  $687\text{-m}\mu$  fluorescence band. This green pigment appears to be very similar to bacterioviridin of the green photosynthetic bacteria, and it may be normally a minor constituent of purple bacteria or may be a decomposition product formed during extraction.

## Fluorescence Spectra of Pigments in Live Plants

### Chlorophyll *a*

The fluorescence spectrum of chlorophyll *a* in ether is compared in Fig. 10 with that of chlorophyll *a* in a very thin layer of plant material, sea lettuce (*Ulva*). The fluorescence spectra, like the absorption spectra, of chlorophyll pigments are shifted toward shorter wavelengths when taken out of the plant by organic solvents. The extent of this shift is, however, different in plants which have been grown in the light and in etiolated albinos that have very recently had their chlorophyll formed from protochlorophyll by exposure to light. Table I shows that the freshly formed chlorophyll *a* has its peak between

TABLE I  
Wavelengths of the Chlorophyll *a* Fluorescence Peak

State of Chlorophyll		Wavelength, $m\mu$
In solution	Ether	668
	Olive oil <sup>a</sup>	672
	Methanol <sup>a</sup>	674
In etiolated albino plants just after transformation from protochlorophyll	Barley <sup>b</sup>	675-677
	Corn	676
In pale light-grown plants	Barley <sup>b</sup>	684
	Albino ivy leaf	681-682
	Albino grape leaf <sup>b</sup>	680
	<i>Ulva</i>	681-683

<sup>a</sup> Zscheile and Harris, 1943.

<sup>b</sup> Todd, Virgin, and El Wakeel, unpublished.

that of chlorophyll in solvents and of chlorophyll in light-grown plants. Perhaps this freshly formed chlorophyll is not yet completely built into its normal state in nature as a protein complex.

Now we will see what happens to the fluorescence spectrum of chlorophyll in living material when it is present in higher concentrations. Figure 11 compares the fluorescence spectrum of a normally green grape leaf with that of a white grape leaf that is apparently completely devoid of chlorophyll, as far as one can see by eye or by the

microscope. In the normal leaf the much higher concentration of chlorophyll gives an increase in the height of the 730-m $\mu$  fluorescence band which is not absorbed to any great extent. On the other hand, the height of the main peak at 685 m $\mu$  is nowhere near as high as would be expected from the amount of chlorophyll present. This relative decrease in height is due to selective reabsorption of the fluorescent light near the chlorophyll absorption band by chlorophyll itself. The incident blue light does not penetrate very deeply into

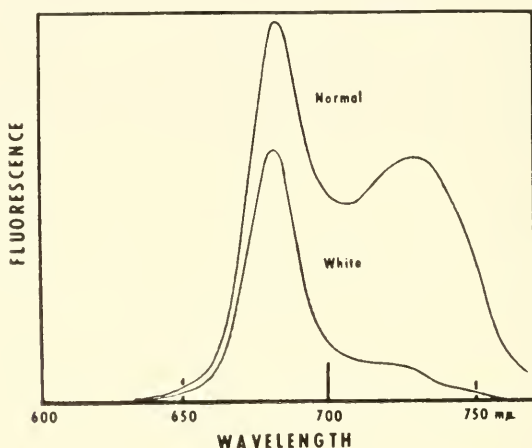


FIG. 11. Fluorescence spectra of chlorophyll *a* in an albino and in a normal grape leaf. (El Wakeel, Virgin, and Todd, unpublished.)

the leaf. If a very dark green leaf is illuminated with blue light, as in Fig. 12, we find a spectrum which is greatly distorted, but nevertheless the two peaks are clearly recognizable. If, however, this same leaf is illuminated with green light which penetrates the leaf, then the fluorescence that is emitted within the leaf is largely reabsorbed on its way out. In this case the deeper penetration leads to a higher emission of light at longer wavelengths, but the main fluorescence peak has practically completely disappeared. The fact that the main peak does not show up is presumably due to the weak absorption of the incident light in the outside layers of the leaf. An alternative possible explanation of the shape of these curves might be that there was another pigment present in the leaf which preferentially absorbed green light and fluoresced at longer wavelengths. It appears

impossible to use such curves to substantiate the existence of other pigments with long-wavelength fluorescence bands, although that would be the more obvious conclusion to be drawn from them. Virgin (1954) has found that the distortion of fluorescence spectra is very strongly influenced by the degree of light scattering within the leaf. This distortion can be greatly reduced by infiltrating the air spaces of the leaf with water.

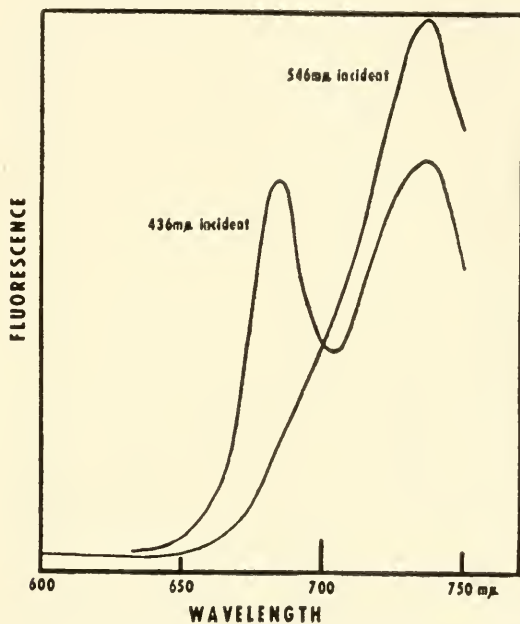


FIG. 12. Fluorescence spectrum of a dark green leaf, *Photinia arbutifolia*, when illuminated by strongly absorbed blue light as compared with weakly absorbed green light. (Hill, Young, and French, unpublished.)

### Protochlorophyll

The fluorescence spectrum of purified protochlorophyll in acetone is illustrated in Fig. 13. The fluorescence spectrum of something which might be called a chloroplast preparation from etiolated barley is also given. This curve bothered us greatly for some time, because the measurements of the action spectrum for the transformation of protochlorophyll to chlorophyll (Koski, French, and Smith, 1951)

showed that the absorption maximum of protochlorophyll in barley was at  $650\text{ m}\mu$ . The fluorescence spectra are normally at longer wavelengths than the absorption maximum. This discrepancy is now satisfactorily cleared up. It is due to an effect which is shown in Fig. 14, the fluorescence spectrum of the inner seed coat of banana squash. There is a fluorescence peak at about  $635$  and another one somewhat beyond  $650\text{ m}\mu$  while the major peak comes at about  $700\text{ m}\mu$ . Offhand,

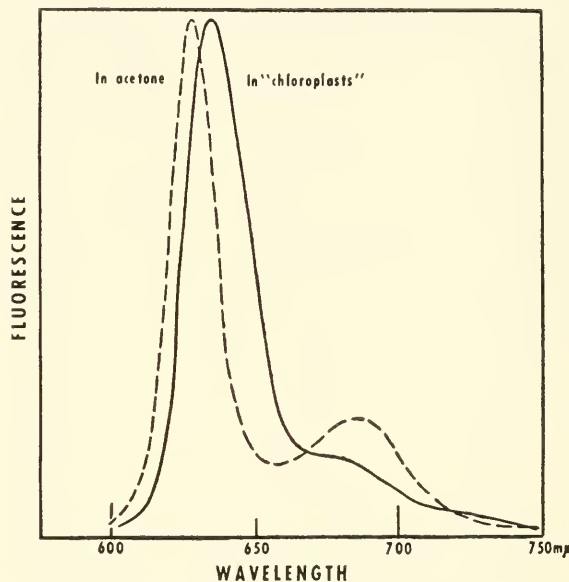


FIG. 13. Fluorescence spectrum of protochlorophyll in acetone and in a heated water suspension of etiolated barley leaf macerate. (Smith, unpublished.)

this looks like a beautiful composite curve for a mixture of three pigments. The acetone extract, however, gives a fluorescence curve for pure protochlorophyll. The residue, after acetone extraction, shows a trace of protochlorophyll fluorescence and nothing else. The absorption curve of the extract agrees with that of protochlorophyll. Here we have an excellent illustration of data which can be most misleading. The squash seed coat is a very highly colored material in which intense reabsorption of the fluorescent light takes place. The  $705\text{-m}\mu$  peak we believe to be merely the unreabsorbed minor long-wavelength band of protochlorophyll. The other peaks are the major



fluorescence peaks of protochlorophyll which may exist in the plant in two forms—one having an absorption peak at 635, the other at about 650  $m\mu$ . This explanation would be hard to believe were it not for the recent experiments of Dr. Smith in which he has clearly shown by absorption measurements the conversion of the 650- $m\mu$  form in freshly ground dark-grown barley leaves suspended in glycerin to the 635- $m\mu$  form in suspensions that have been heated or allowed to stand.

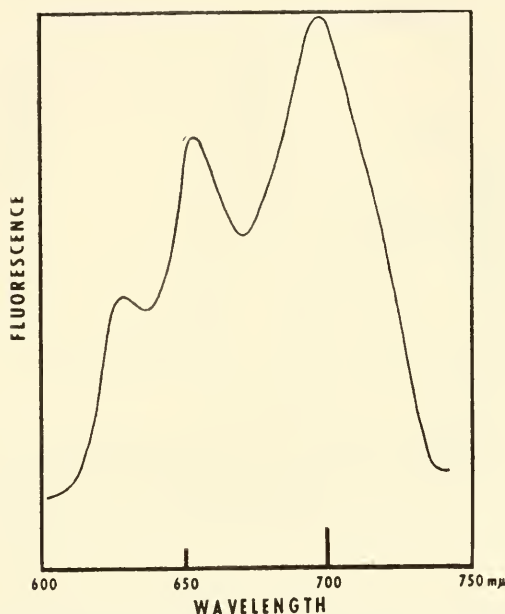


FIG. 14. Fluorescence spectrum of the inner seed coat of a banana squash. (Hill, Smith, and French, unpublished.)

The fluorescence spectrum of a dark-grown leaf exposed to light for a short period of time is given in Fig. 15. Here we can clearly distinguish the fluorescence spectrum of protochlorophyll in the presence of a considerably larger amount of chlorophyll. Dr. Hemming Virgin is at present looking into the possibility of using such curves for the quantitative measurement of rates of protochlorophyll transformation to chlorophyll in living leaves. He has found that much more reproducible results may be obtained by infiltrating the leaves

with hot water before measurements. It is of course necessary to heat the leaves to prevent further transformation by the light used for exciting the fluorescence and this heating itself shifts the fluorescence peak of protochlorophyll from 655 to about 638  $m\mu$ .

### Chlorophylls *b* and *c*

The fluorescence band of chlorophyll *b* at 655  $m\mu$  in several different species of plant has been reported (Rabinowitch, 1951, p. 807). This

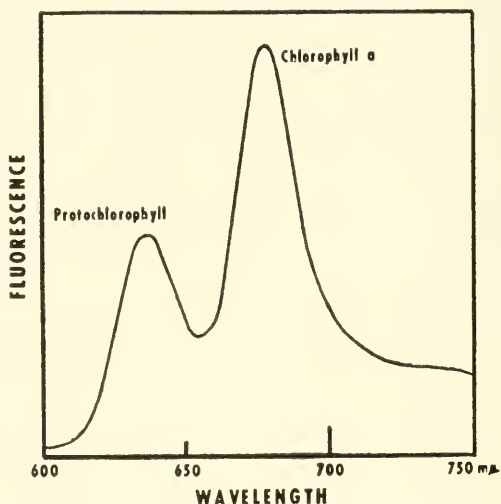


FIG. 15. Fluorescence spectrum of a partially greened barley leaf. (Young, unpublished.)

band has never been found in any of our curves of living plants. We have measured very pale leaves with the hope of finding chlorophyll *b* fluorescence which might have been reabsorbed in more highly colored material, but it has never appeared. There are several possible reasons for its lack. One may be that chlorophyll *b* is appreciably less fluorescent and that it occurs in smaller amounts than chlorophyll *a*. Another reason may be that the efficiency of energy transfer from chlorophyll *b* to chlorophyll *a* is very high. This transfer has been found by Watson and Livingston (1950), Young (1952, Carnegie Institution Year Book No. 51), and Duysens (1952) in ether solutions of the mixed pigments. A less interesting but also possible reason may

be that the chlorophyll *b* fluorescence band is near enough to the absorption maximum of chlorophyll *a* to be strongly reabsorbed.

The fluorescence spectrum of two suspensions of the diatom *Nitzschia*, kindly given us by Professor C. B. van Niel, which is presumed to contain chlorophyll *c*, is shown in Fig. 16 as compared with the fluorescence spectrum of the white part of an ivy leaf.

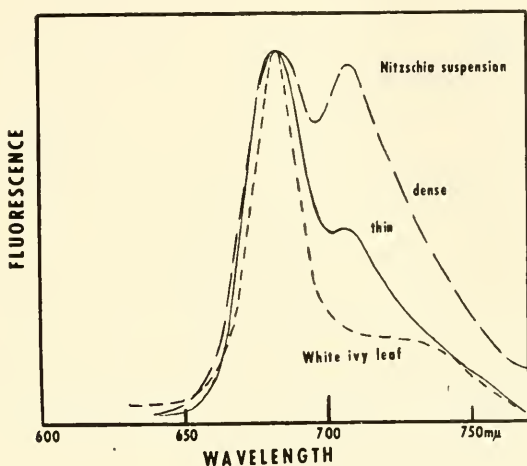


FIG. 16. Fluorescence spectra of a thick and a thin suspension of *Nitzschia closterium, minutissima*, as compared with that of chlorophyll *a* in the white part of a variegated ivy leaf. The diatom culture was kindly given us by Professor C. B. van Niel.

Instead of finding a chlorophyll *c* band at the anticipated position of approximately 640 mμ, a new band appeared at about 705 mμ. The cause of this 705-mμ fluorescence is not known. One possible interpretation might be that it is indeed due to chlorophyll *c*, but it is the longer wavelength subsidiary band which could reasonably be expected to be at about this position and that the absence of the main band, anticipated at about 640 mμ, is due to its reabsorption by chlorophyll *a* within the diatoms. The cells themselves are quite dark so that very strong reabsorption of fluorescence, even within a single cell, might not be inconceivable. The action spectrum for the excitation of fluorescence in this material, Fig. 17, confirms again the participation of light absorbed by fucoxanthin in the excitation of

chlorophyll fluorescence. It also shows that other carotenoids absorbing at shorter wavelengths than fucoxanthin are less effective in transferring energy to chlorophyll.

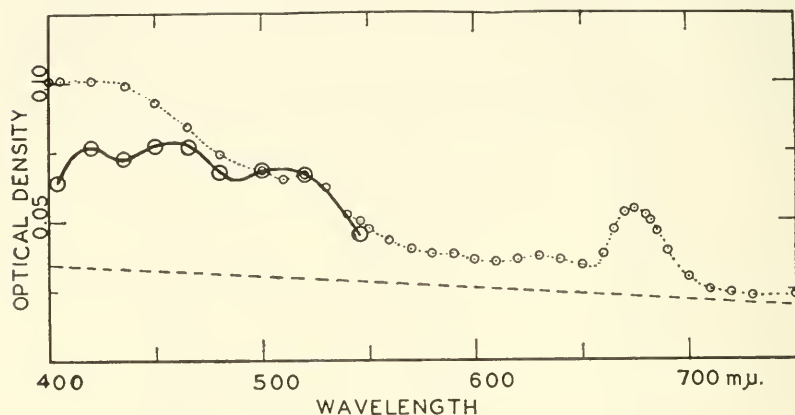


FIG. 17. The absorption spectrum of the thin suspension of *Nitzschia* in dilute agar (small circles), as compared with the action spectrum for fluorescence excitation (heavy line). The lower straight line is the estimated contribution of scattering to the measured absorption; it was used as the baseline above which the action spectrum was plotted.

### Phycobilins and Chlorophylls in Red Algae

The spectra of red algae demonstrate some of the more complex fluorescence phenomena. They contain the fluorescent pigments phycoerythrin and phycocyanin in addition to chlorophylls *a* and *d* so that fluorescence spectra of mixtures are obtained.

Haxo and Blinks (1950) have found that the light absorbed by phycoerythrin and phycocyanin is used in photosynthesis even more efficiently than that absorbed by chlorophyll. Do these accessory pigments act directly in photosynthesis or do they participate by transferring their energy to chlorophyll *a*, which seems to be a more or less universal photosynthetic pigment? The fluorescence spectra for various incident wavelengths of measured intensity from *Porphyridium cruentum* are given in Fig. 18. We see the typical peaks due to phycoerythrin, 678  $m\mu$ , phycocyanin, 655  $m\mu$ , and chlorophyll *a*, at 685  $m\mu$ . Figure 19 shows the analysis of a similar curve in terms of the

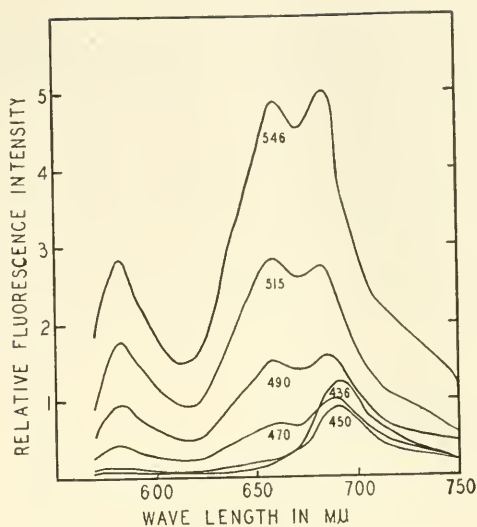


FIG. 18. Fluorescence spectra of *Porphyridium cruentum* excited by various incident wavelengths. The curves are reduced to their relative sizes for the same number of incident quanta at all wavelengths. (French and Young, 1952.)

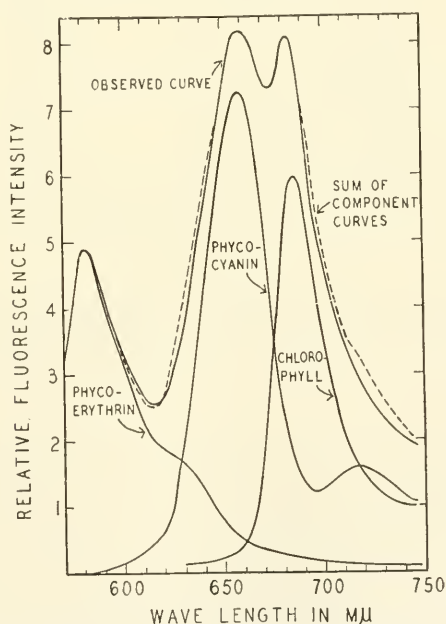


FIG. 19. Estimated contribution of three different pigments to the total fluorescence of *Porphyridium* when illuminated by wavelength 530  $m\mu$ . (French and Young, 1952.)

fluorescence contributed by each of the three pigments. A similar dissection of these curves and of five more of the same set with different incident wavelengths was carried out by means of a graphical computer (French *et al.*, 1954) so that we could calculate the effectiveness of each wavelength in exciting fluorescence of each of the three pigments separately. A plot of the effectiveness of different wavelengths in exciting chlorophyll fluorescence is given by the circles in Fig. 20, which also shows the absorption spectra of chloro-

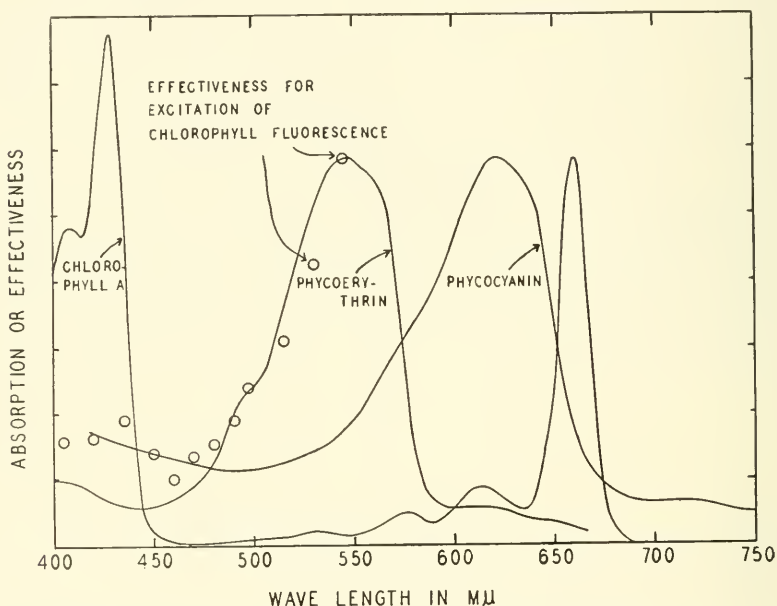


FIG. 20. Action spectrum for the excitation of chlorophyll *a* fluorescence in *Porphyridium* compared with the absorption spectra of water solutions of phycoerythrin and phycocyanin and on ether solution of chlorophyll *a*. (French and Young, 1952.)

phyll *a*, of phycoerythrin, and of phycocyanin. There is some fluorescence by direct absorption of blue light by chlorophyll *a*, but strangely enough the points rise along with the phycoerythrin absorption curve in the green part of the spectrum where chlorophyll absorbs weakly. This shows clearly that phycoerythrin is an effective light absorber for the excitation of chlorophyll fluorescence. Furthermore, the phycocyanin fluorescence, as well as that of phycoerythrin, also



follows the same type of excitation curve, except that these two drop to a low minimum at about  $435\text{ m}\mu$ . It therefore appears in agreement with Duysens (1952) that phycoerythrin absorbs energy and transfers it to the phycocyanin which passes it along to chlorophyll. Presumably this same process may be followed by the energy used for photosynthesis, although this conclusion of course involves several assumptions.

The effect of the intensity incident on a red alga upon the shape of the fluorescence curve is shown in Fig. 21. These curves were meas-

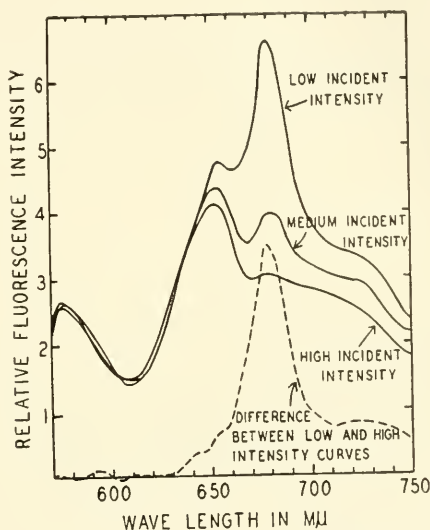


FIG. 21. Fluorescence spectra of a red alga after illumination for 6 minutes with three different intensities. Only the chlorophyll peak is reduced in height by prolonged illumination at high intensity. (French and Young, 1952.)

ured, as were all the others in this article, after the initial changes of intensity of fluorescence, the Kautsky effect, had come to completion, so that constant readings were obtained. These changes are connected with the induction period of photosynthesis. A great deal of speculation has been aroused by observations of these phenomena. Here we see that low intensity gives far more chlorophyll fluorescence in proportion to that of phycoerythrin and phycocyanin than do higher intensities. This is another manifestation of the Kautsky effect. Here

it is evident that it is only the chlorophyll fluorescence and not the fluorescence of phycoerythrin and phycocyanin that is influenced by the Kautsky effect. It thus appears that the products of photosynthesis which somehow or other quench fluorescence act only upon chlorophyll, either because only chlorophyll is sensitive to such quenchers or because the immediate products of photosynthesis are more closely associated with chlorophyll than with the other pigments.

### A New Leaf Pigment Found by Fluorescence

A paper by Goodwin, Koski, and Owens (1951) describes a new leaf pigment, traces of which are present in the cells surrounding the

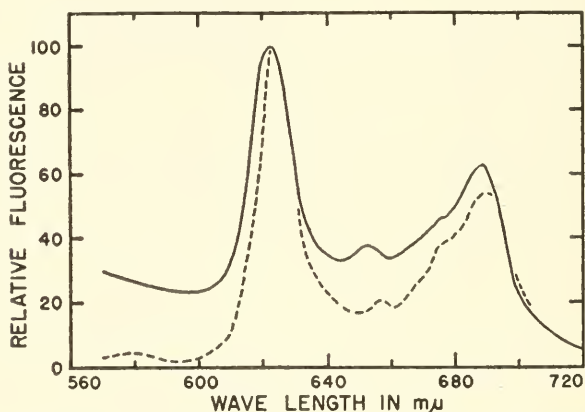


FIG. 22. Fluorescence of the methanol extracted epidermis of *Vicia* (solid line), compared with uroporphyrin I octamethyl ester in methanol (dotted line). (Goodwin, Young, and Owens, 1952.)

guard cells of certain species of *Vicia*. This pigment was discovered with a fluorescence microscope and was reasonably well identified solely by fluorescence spectroscopy. The guard cells making the stomata contain chlorophyll and show a red fluorescence. In the cells around them there are small bodies having a somewhat more orange fluorescence. A little piece of the epidermis peeled off from the rest of the leaf shows chlorophyll fluorescence and also another peak at approximately 615 mμ. Some of this epidermis was treated with methanol to remove the chlorophyll, but the new fluorescing pigment was not extracted by methanol. Figure 22 shows the fluorescence spectrum of

the epidermis wet with methanol after extraction of the chlorophyll. In comparison with this is also drawn as a dotted line the fluorescence spectrum of uroporphyrin-1-octamethyl ester showing a rather close agreement. The fluorescence spectrum of a solution of the pigment in hydrochloric acid also agreed with that of a hydrochloric acid solution of this porphyrin.

Now it is well known that porphyrins generally have a very intense absorption band in the blue part of the spectrum known as the Soret band. The trace amounts of this new fluorescent pigment material and its insolubility made it impossible to measure its absorption spectrum. However, the effect of various wavelengths in exciting fluorescence of the pigment in epidermal peels was measured. This action spectrum showed points of equal height at about 405 and 410  $m\mu$  and dropped to lower values on both sides. By interpolation it was found that the Soret band of this pigment whose absorption could not be measured directly is at 408  $m\mu$ .

#### ACKNOWLEDGMENTS

Extended collaboration with Drs. Violet K. Young, James H. C. Smith, and Hemming I. Virgin produced most of the results here described. Shorter periods of investigations with Drs. Robert Hill and Glenn Todd opened up new aspects of the field. The electronic components of apparatus were designed and constructed by Messrs. Bertram G. Ryland, Henry C. Patton, Jr., George H. Towner, and Dr. Donald R. Scheuch. The mechanical parts of the monochromators were built by Mr. Frank Schuster. The replica transmission grating was made available by Professor R. W. Wood, and the original reflection grating by Mr. Harold Babcock.

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# Kinetics of Chemiluminescence of the 2,3-Dihydrophthalazine-1,4-diones

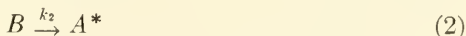
PAUL C. WILHELMSSEN,<sup>\*</sup> RUFUS LUMRY,<sup>†</sup> and HENRY EYRING  
Department of Chemistry, University of Utah, Salt Lake City, Utah

The purpose of this paper will be to present some new data from experiments on the chemiluminescence of the 2,3-dihydrophthalazine-1,4-diones (DPD) and in particular the 5-amino derivative (luminol). In addition, a brief discussion will be given of the known facts on which a mechanism for the chemiluminescence of the DPD's must be based. A tentative mechanism will be proposed which explains the available facts and points out possible directions for further investigation.

The most important data that this paper will discuss have to do with the kinetics of the reaction. Chemiluminescent reactions such as the oxidation of the DPD's are particularly well adapted for kinetic studies. The intensity of the light emitted by a chemiluminescent reaction is the number of photons ( $I$ ) emitted per second. The number of photons emitted is in turn related to the number of excited molecules  $A^*$  and the rate of emission  $k_1$ :

$$I = k_1 A^* \quad (1)$$

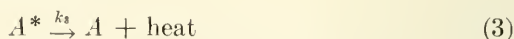
The number of excited molecules is a function of their rate of production



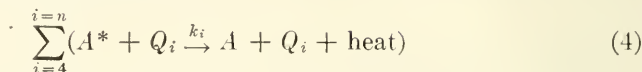
<sup>\*</sup> A research fellowship granted by the University of Utah Research Committee which greatly aided this author's contribution to this work is gratefully acknowledged. The work was partially supported by the United States Atomic Energy Commission.

<sup>†</sup> Present address: Institute of Technology, School of Chemistry, University of Minnesota, Minneapolis, Minnesota.

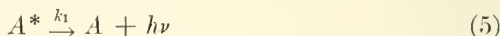
minus the rate of internal quenching



the rates of external quenching



and the rate of emission of light



The concentration of excited molecules as a function of time is given by

$$\frac{dA^*}{dt} = k_2B - (k_1 + k_3 + \sum_{i=4}^{i=n} k_i Q_i) A^* \quad (6)$$

The fraction of the excited molecules that produce light is

$$\phi = \frac{k_1}{k_1 + k_3 + \sum_{i=4}^{i=n} k_i Q_i} \quad (7)$$

This fraction is equal to the rate of emitting light divided by the total rate of destruction of the excited molecules. If one knows the relationship between these various reaction rates, it is possible to relate the intensity of the light to the rate of production of excited states and therefore to the rate of the reaction. However, for the purpose of this paper, it is assumed that  $\phi$ , the fraction of excited molecules that produce light, is independent of the concentration and nature of the oxidants employed. This would be true as long as the principal mode of quenching is by means of intramolecular transfer of energy or when the concentrations of the external quenchers remain constant. The effect of temperature on  $\phi$  will be determined by an experimental study of the quantum yield of a particular reaction as a function of temperature.

### Historical

The DPD's and, in particular, luminol are well suited for kinetic studies. The light emitted on oxidation is relatively bright and luminol at least is readily available. Because of the availability of luminol,



most of the kinetic studies have been done on this compound. However, there is evidence that the mechanism is the same in each case, and most information concerning the reaction of one of the DPD's can be extended to the remainder. This similarity has been found true in the investigations of Zellner and Dougherty (1937) where it was found that the rate of oxidation of many of the DPD's is the same when measured by the rate of evolution of nitrogen. The apparent difference in rates, when the intensities of the luminescence are compared, is probably due largely to the different efficiencies of the emitters produced in each case. An additional possibility is that there are two or more reaction routes and that different fractions of some of the DPD's proceed by the luminous and nonluminous reactions, in which case some forms of information about one of the DPD's could not be simply extended to the others in the series. Further investigations are needed in order to determine the extent to which this second possibility must be considered.

### The Role of Oxygen

Many different substances and procedures have been employed in connection with the DPD's to produce chemiluminescence. Frequently, hydrogen peroxide is used either alone or with various other agents such as hemin, potassium ferricyanide, or sodium hypochlorite (Albrecht, 1928). Chemiluminescence can also be produced by adding solutions such as ferricyanide or hypochlorite to an alkaline solution of the DPD. Some investigators have employed electrolysis (Harvey, 1929; Spruit, 1950; Bremer, 1953) and supersonics (Prudhomme, 1949; Flosdorf *et al.*, 1936). However, in the final analysis, either oxygen or a compound that readily decomposes to form oxygen, e.g., hydrogen peroxide or sodium hypochlorite, appears to be indispensable. It might seem that the oxidation of the DPD's by ferricyanide does not appear to require oxygen. However, as shall shortly be seen, the chemiluminescent reaction is largely dependent on dissolved oxygen.

Inasmuch as oxygen is so intimately involved in the chemiluminescence of the DPD's, it will be interesting to study the reaction between oxygen and luminol. Some authors (Harvey, 1929; Drew, 1938) have reported that no light was observed when oxygen was bubbled through a luminol solution. Their failure to observe the light

was probably due to lack of suitable light detection equipment and improper choice of concentrations. With the proper concentrations of reagents, it is possible to produce a light visible to the dark-adapted eye. A solution of one gram of luminol per liter of 2N NaOH has been found satisfactory in this connection although other concentrations will also produce light, especially when heated. This reaction between luminol and molecular oxygen has been studied briefly by Sveshnikov (1938), Bernanose *et al.* (1947), and Bremer (1953). However, the results contained in this paper are the first extensive kinetic studies that have been carried out on this interesting reaction.

Some qualitative aspects of the reaction involving oxygen and the importance of dissolved oxygen on the emission of light in the case of oxidation by ferricyanide are shown in the following experiment.

### Experimental

A series of A tubes was arranged as in Fig. 1. The first A tube arm contained a solution of potassium ferricyanide, the second arm con-

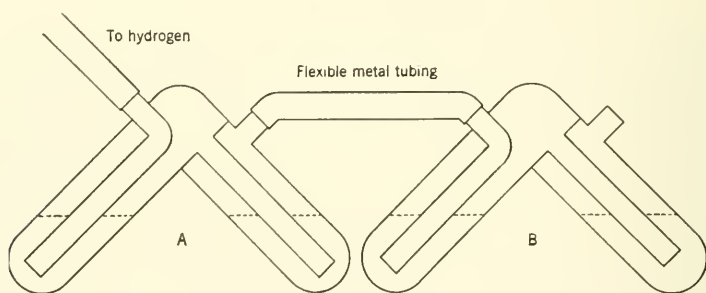


FIG. 1.

tained a solution of 0.05M luminol in 0.1N NaOH. The first arm of the second A tube contained a dilute suspension of luminous bacteria such as *A. fischeri* or *P. phosphoreum*<sup>\*</sup> in a glucose media. The second arm of the second A tube was filled with water. The arm containing luminol was heated to 60° C. A readily visible light was produced by the luminol solution that was about ten times as bright as the luminous bacteria. However, when the oxygen was removed by flushing

<sup>\*</sup> These bacteria were kindly furnished by Dr. Frank Johnson.

the system with oxygen free hydrogen, the light from the luminol was extinguished. It required less than a minute of bubbling hydrogen through the system before the light from the luminol faded below the visible threshold. The luminous bacteria continued to glow for a couple of minutes after the luminol ceased luminescing. This indicates that a higher concentration of oxygen is required by the luminol to produce a visible light than is required by luminous bacteria.

If the ferricyanide solution is now added to the luminol solution by tilting the A tube, the bright chemiluminescence characteristic of the reaction luminol and ferricyanide when saturated with air is produced. An hour of bubbling hydrogen through the solutions did not visibly affect the intensity of the light produced by luminol and ferricyanide. This means that a smaller quantity of oxygen is required in this case than is used with luminous bacteria—a very small quantity indeed considering that a partial pressure of 0.0007 mm Hg of  $O_2$  is all that is required by bacteria (Harvey and Morrison, 1923). However, when the hydrogen was bubbled through the system for twenty-four hours, the light produced by the addition of ferricyanide to luminol became faint and of short duration. Bremer (1953) performed a similar group of experiments where the oxygen was removed by repeated freezing of the solution and evacuation of the space above. She has reported results similar to those just described.

The small residual amount of light produced with luminol and ferricyanide in the supposed absence of oxygen is possibly due to a small trace of oxygen still remaining. However, Bremer (1953) was not able to extinguish the residual glow by extended treatment with repeated freezing and evacuation of the system, and the present authors found no difference in the light obtained when the hydrogen was passed through the system for one day or for five days. The hydrogen employed in these experiments was purified by passing over platinized asbestos and might conceivably have contained a small trace of oxygen. The existence of a second, less efficient mode of chemiluminescence is the alternative. Another line of evidence that makes it troublesome to exclude the existence of this less efficient reaction comes from another source.

It has been found that hydrazine and ferricyanide give out a feeble

but visible light.\* The intensity of the chemiluminescence of hydrazine appears to be independent of oxygen concentration. This is interesting as luminol and the DPD's in general can be thought of as substituted hydrazine. This being the case, it should not be surprising if both were to undergo many of the same reactions, which suggests that the DPD's undergo a similar weakly luminous reaction with ferricyanide in the absence of oxygen.

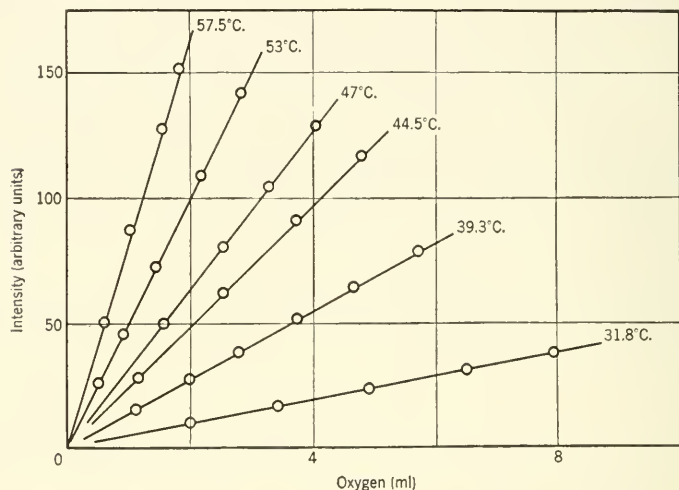


FIG. 2. Intensity versus time curve for luminol being oxidized by ferricyanide in the presence of oxygen.

It was found by Bremer (1953) that if oxygen was admitted after the ferricyanide and luminol have been allowed to react under anaerobic conditions, the solution would emit a reasonably bright light. The present authors have been able to confirm this observation and, in addition, have found that if a few minutes is allowed to elapse between the mixing of the luminol and ferricyanide, no light is produced. The importance of Bremer's observation will be discussed later in connection with the proposed mechanism. The second observation is important in that it indicates that ferricyanide does react with luminol in the absence of oxygen. This is proof that there is at least one other reaction route besides the chemiluminescent one involving oxygen. The experiments just described indicate that, in any case,

\* Unpublished work of the present authors.

very small quantities of oxygen greatly affect the efficiency of the chemiluminescence.

A difference in the rates of the reaction with oxygen in the presence and absence of an additional oxidant might be inferred from the difference in quantities of oxygen required to produce a visible light in each case. However, a better idea of the relative rates might be obtained from a study of the length of times required for the reactions to proceed to a certain fraction of completion. The reaction involving only dissolved oxygen requires weeks or months to proceed even a small fraction of the way to completion. The reaction where an additional oxidant such as ferricyanide is present is essentially complete in a matter of minutes. Figure 2 shows a typical plot of intensity versus time for a solution that was 0.05*M* luminol, 0.005*M* ferricyanide and 1.0*N* NaOH.

The kinetic study of the reaction between the DPD's and oxygen would at best be very time consuming if the reaction were carried to completion. However, as was stated earlier, the reaction rate can be related to the intensity of the chemiluminescence if the temperature dependence of  $\phi$  is known. An experimental determination of this temperature dependence will now be discussed.

### Experimental Temperature Dependence of $\phi$

The reaction between ferricyanide and luminol in the presence of oxygen was chosen for a study of the temperature dependence of  $\phi$  because of its relative freedom from complications and the ideal speed of the reaction for kinetic studies.

Measurements were made by using the equipment diagrammed in Fig. 3. The solution of luminol and oxygen was mixed with the ferricyanide solution by means of a thermostated mixer, 5, similar to the one employed by Chance (1940). The solutions were thermostated and stored under pressure in flasks 2 and 3, and when the double stopcock 4 was opened, the solutions rapidly mixed and flowed along a 1-mm capillary. The solutions were allowed to flow until the light emitted reached a steady value. The flow was then stopped and the intensity of the light from a small quantity of solution in a short section of the capillary was studied. The light was measured by a photomultiplier, 7, which was in turn connected with an oscilloscope,



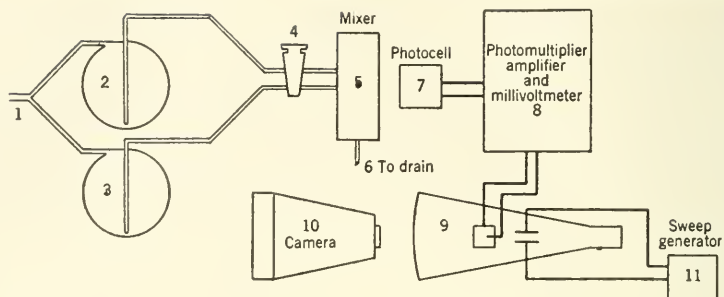
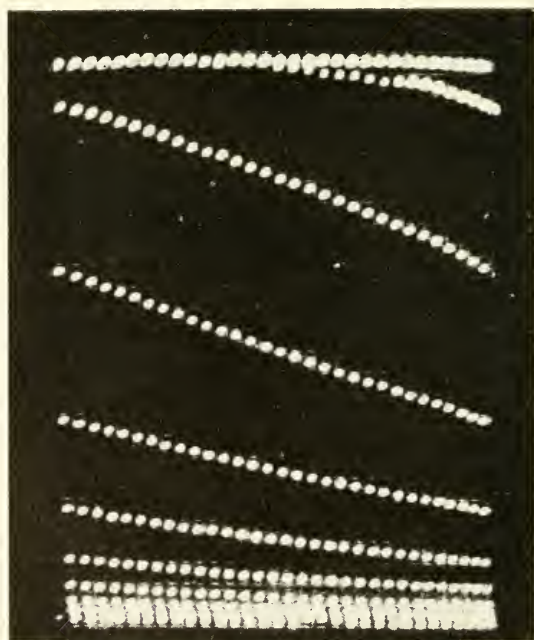


FIG. 3.

9. In this manner, the intensity of the light was recorded as a vertical deflection and time was measured by means of the sweep along the  $x$  axis. A typical oscilloscope trace is given in Fig. 4. The final portion of the reaction is quite slow and was followed by periodic readings with a millivoltmeter 8. A typical intensity versus time curve is given in Fig. 3. Graphical integration of the total quantity of light emitted

FIG. 4. Typical oscilloscope trace. The dots are  $\frac{1}{60}$  second apart.



was carried out on several runs made at different temperatures, but with the same initial concentrations of reagents. These values are given in Table I and are plotted in Fig. 5.

The variation in the quantity of light emitted was assumed to be proportional to the change in  $\phi$ . To obtain an equation for the

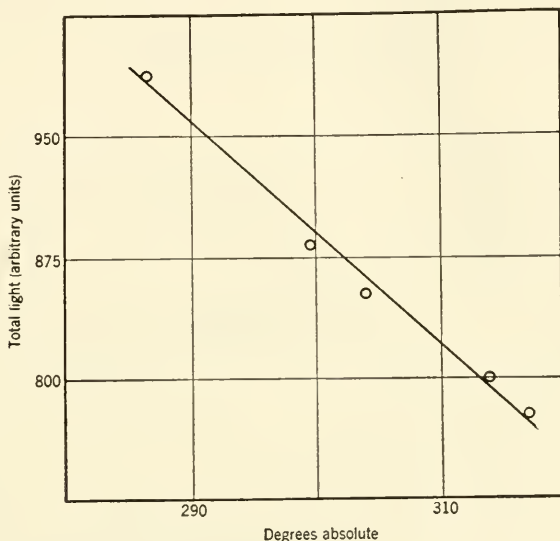


FIG. 5. Total light versus temperature.

temperature dependence of  $\phi$  it is necessary to return to equation 7.

If it is assumed that one particular mode of quenching is the principal one, equation 7 becomes

$$\phi = \frac{k_1}{k_1 + k'} \quad (8)$$

The rate of emission of light by an excited molecule is usually independent of temperature and therefore any temperature effect will generally be on  $k'$ . The rate  $k'$  is given by the rate equation

$$k' = \frac{\kappa k T e^{-\Delta H^\ddagger/RT} e^{\Delta S^\ddagger/R}}{h} \quad (9)$$

TABLE I

$\frac{d \ln I}{dt}$	$k'$	$T_{\text{abs}}$	$\frac{k'}{T}$	$\ln \frac{k'}{T}$	$\frac{1}{T} \times 10^3$	$\int I dt$	$\phi$	$10 \frac{1-\phi}{\phi T}$	$\ln \left( \frac{1-\phi}{\phi T} \right) 10$
0.189	189	286.5	0.66	-0.42	3.50	1027	0.110	2.83	1.075
0.248	248	298.5	0.83	-0.19	3.35	895	0.096	3.16	1.150
0.274	274	304.3	0.90	-0.11	3.28	860	0.092	3.24	1.175
0.314	312	313.8	1.00	0.00	3.19	795	0.085	3.40	1.225
0.333	333	316.9	1.05	0.05	3.16	765	0.082	3.52	1.230

Luminol =  $10^{-5} M$ ; ferricyanide =  $10^{-3} M$ ; oxygen =  $10^{-4} M$ .

Substituting this expression for  $k'$  into equation 8 gives

$$\phi = \frac{k_1}{k_1 + \kappa \frac{kT}{h} e^{-\Delta H^\ddagger/RT} e^{\Delta S^\ddagger/R}} \quad (10)$$

Rearranging and taking the logarithm of this expression gives

$$-\frac{\Delta H^\ddagger}{RT} = \ln \left[ \frac{1 - \phi}{T\phi} \frac{k_1}{\kappa \frac{k}{h} e^{\Delta S^\ddagger/R}} \right] \quad (11)$$

The terms  $k_1$ ,  $k$ ,  $h$ ,  $\kappa$ , and  $e^{\Delta S^\ddagger/R}$  either are or may be assumed to be temperature independent. The plot of  $\ln(1 - \phi)/T\phi$  versus  $1/T$  can be seen to give  $-\Delta H^\ddagger/R$ . However, in order actually to determine  $\Delta H^\ddagger$ , absolute values are needed for  $\phi$  at the different temperatures. These values have not been determined for the emitter in the case of the chemiluminescence of luminol. However, the value obtained for  $\Delta H^\ddagger$  for small  $\phi$  is proportional to the logarithm of  $\phi$ . This might justify making an approximation of  $\phi$  at some particular temperature and using it to calculate values at other temperatures.

A lower limit for  $\phi$  in the case of luminol would be 0.003 which is the observed overall quantum yield for the chemiluminescence of luminol (Harris and Parker, 1935; Bernanose, 1951; and Bremer, 1953). Using this value at 20° C and making the appropriate calculations lead to a value of 2.6 kcal for  $\Delta H^\ddagger$ . This value assumes that all the luminol molecules become excited and ignores the existence of side reactions.

Another value for  $\phi$  at 20° C which might be more reasonable is 0.1 which is based on the observed fluorescence yields of luminol (Spruit, 1950). Although the use of this value is open to the objection that the emitting molecules are not the same in the two cases, the molecules are probably similar and the fluorescence yield of a molecule is essentially  $\phi$ . Values are tabulated in Table I for  $\phi$ 's based on  $\phi$  being 0.10 at 20° C. The use of this group of  $\phi$ 's give a value of 1 kcal for  $\Delta H^\ddagger$ . The value of 1 kcal and the value 0.1 for  $\phi$  at 20° C gives an expression

$$\phi = \frac{1}{1 + 0.169T e^{1000/R T}} \quad (12)$$

for the temperature dependence of  $\phi$  which fits the experimental data reasonably well.

An alternative explanation for the variation in the efficiency of light production is that there is a change in the fraction of molecules passing along the reaction route favorable to light production. Inasmuch as there is only a relatively small change in the quantity of light emitted as the temperature is changed, the error in assigning the entire effect to changes in  $\phi$  will be small in the case yet to be considered.

Although the temperature control was not too good in these experiments, it was possible to obtain an approximation of the rate as a function of temperature by noting the time for the intensity to drop to various fractions of the initial intensity. That is,

$$-\frac{dX}{dt} = k'[X][Y] \quad (13)$$

where  $Y$  is the concentration of the species that remains essentially constant, which in this case is the ferricyanide, and  $X$  is the concentration of luminol. Equation 13 can be rewritten as

$$-\frac{d \ln X}{dt} = k'[Y] \quad (14)$$

The quantity  $d \ln X/dt$  is equal to  $d \ln I/dt$  where  $I$  is the intensity of the light in as far as  $\phi$  is independent of concentrations. Values for  $d \ln I/dt$  are given in Table I.

If the standard state is taken as 1 mole per liter, the values for  $d \ln I/dt$  divided by the concentration of the ferricyanide will give values for  $k'$ . These values are also tabulated in Table I.

The rate equation for  $k'$  is

$$k' = \kappa \frac{kT}{h} e^{-\Delta H^\ddagger/RT} e^{\Delta S^\ddagger/R} \quad (9)$$

If this is divided through by  $T$  and the logarithm is taken, the equation becomes

$$\ln \frac{k'}{T} = \ln \kappa \frac{k}{h} - \frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} \quad (15)$$

As can be seen, a plot of  $\ln k'/T$  versus  $1/T$  should give a straight line

of slope  $-\Delta H^\ddagger/R$ . The value obtained from Fig. 6 for  $\Delta H^\ddagger$  is about 3 kcal. Substituting of this value into equation 15 and assuming  $\kappa$  is equal to 1 yields an entropy of activation. The value obtained at 20.3° C is 38 e.u. The standard state used for calculating the entropy in this case was 1 mole per liter.

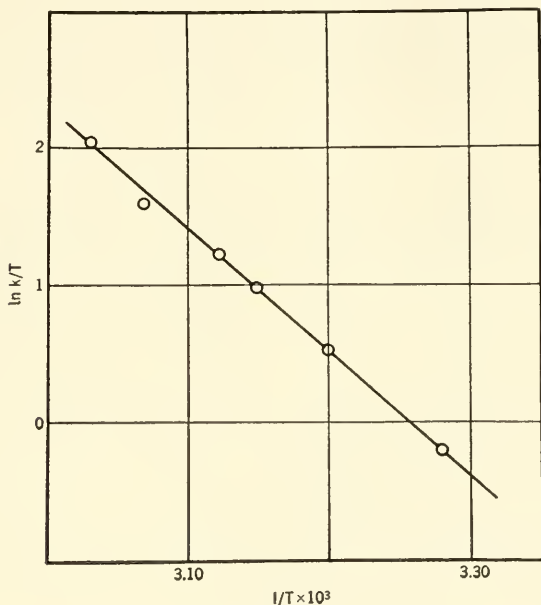


FIG. 6.

### Kinetics of Reaction Involving Oxygen as Oxidant

#### Equipment

The investigations of the kinetics of the reaction involving only oxygen as the oxidant were made with the apparatus of Fig. 7.

The reaction vessel 1 was a 3000-ml round bottom flask with a graduation at the 3000-ml mark. It was placed in heating jacket 2 so that the temperature could be varied at will. The reaction vessel was stirred with a magnetic stirrer, 3. The flask was fitted with two connections, one of which was to the flask containing oxygen, 6, and the other to a manometer, a dry ice trap, 7, and a vacuum pump.

A flask of known volume was used to add oxygen to the system. Before adding oxygen the entire system was evacuated to as low a pressure as possible. Stopcock 4, connecting the reaction vessel with the vacuum pump, was then shut off, and the system allowed to come to equilibrium with respect to water vapor. Stopcock 5 was then closed, and oxygen was added to the flask 6 and the pressure was noted. By noting the pressure change when 5 was opened, a check could be obtained on the volume occupied by the oxygen in the manometer and reaction vessel. A small correction was found neces-

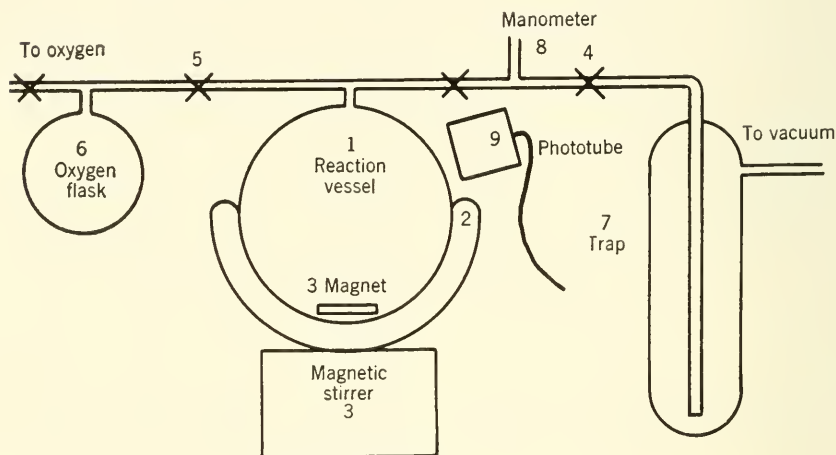


FIG. 7.

sary to compensate for the different volume displaced by the mercury in the manometer as the level changed. The temperature of the reaction solution was measured by a 0.1-degree thermometer.

### Procedure

The distilled water used was boiled to remove as much dissolved oxygen as possible. Sodium hydroxide and luminol were added, and the solution was boiled under reduced pressure to remove as much of the residual oxygen as possible. The solution was concentrated to the correct volume by boiling off the excess water, and the temperature was adjusted. Pure oxygen was added, and the pressure was noted. The solution was stirred by means of the magnetic stirrer, and the rate



of absorption of oxygen was followed by the change in the pressure of the oxygen. Because of the slowness of the reaction, the quantity of oxygen absorbed can be taken as the concentration and can be presumed uniformly distributed throughout the solution.

Various tests were performed to test the validity of the various assumptions. As was stated earlier, the volume of the manometer and reaction vessel was checked by comparison with the vessel used to store oxygen. There was good agreement between the volume determined this way and by actual measurement. Distilled water was

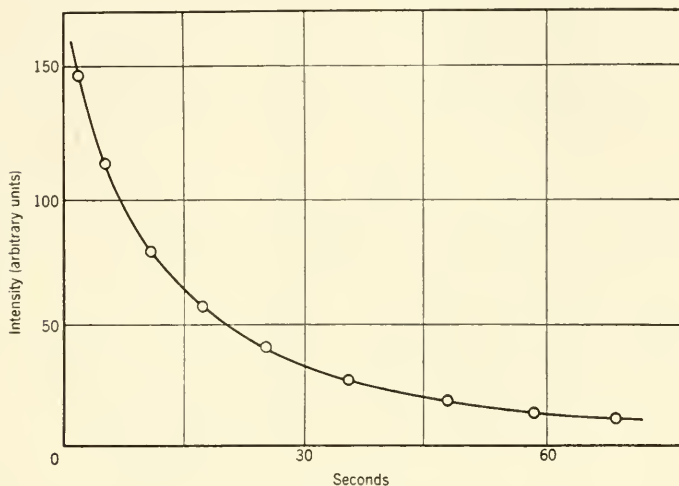


FIG. 8.

added to some of the solutions and the experiment was repeated to check the assumption that the luminol concentration remained essentially constant throughout the experiment. There was no difference in repeated runs greater than the experimental error from other sources. The rate of absorbing oxygen was changed by varying the pressure of the oxygen, but no difference was noted when intensity was related to the quantity of absorbed oxygen.

## Results

Measurements were made relating the intensity and the change in pressure of the oxygen above the reaction solution. The change in

pressure can in turn be expressed in milliliters at S.T.P. of oxygen absorbed. There was a small amount of light even before the oxygen was added. This varied from one experiment to another, depending on the thoroughness of the treatment to eliminate the dissolved oxygen. This was taken to mean that a small quantity of oxygen was still in the solution. The linearity of the plot of oxygen absorbed versus

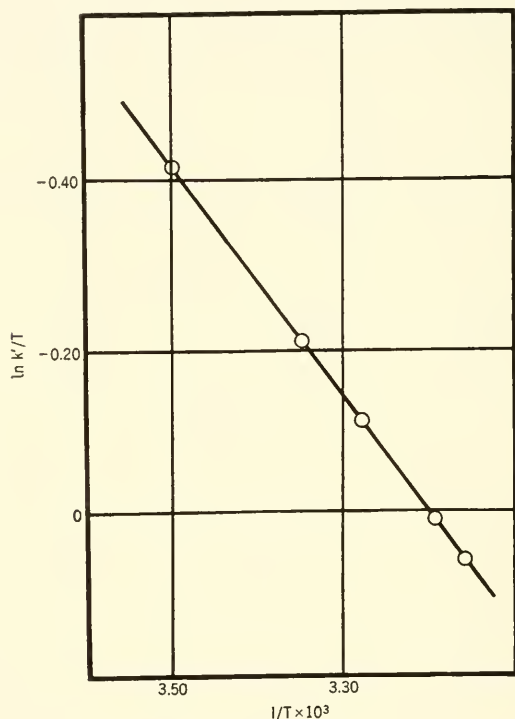


FIG. 9.

intensity permitted the extrapolation to zero concentration of oxygen. These results are given in Fig. 8.

Tests were made at different temperatures. The plots of intensity versus milliliters of  $O_2$  absorbed in Fig. 8 make it possible to read intensities for any given concentration of oxygen as a function of temperature. Equation 12 permits the conversion of intensities into rates. Figure 9 gives the rates for solutions that have absorbed 4 ml

of  $O_2$  corrected to S.T.P. plotted as the  $\ln k/T$  versus  $1/T$ . The slope of this line gives a  $\Delta H^\ddagger$  of 19 kcal using the rate equation. Without some way of obtaining absolute values for  $k'$ , it is impossible to calculate the entropy of formation of the activated complex.

### Information Available about Mechanism

#### Kinetic Data

The data that have been presented are suggestive with respect to the mechanism for chemiluminescence. Inasmuch as the reaction with oxygen alone proceeds very slowly and that with oxygen and an additional oxidant proceeds much more rapidly, it can be reasoned that the mechanism has as its slow step, an oxidation by oxygen which, however, proceeds more rapidly with ferricyanide. Furthermore, it has been demonstrated that oxygen is required for efficient chemiluminescence of the DPD's even when ferricyanide is present. Bremer's experiments, in which she found that light was produced when oxygen was added to the anaerobic mixture of luminol and ferricyanide, might indicate that the initial oxidation precedes the reaction with molecular oxygen (1953). The kinetic data also indicate that the reaction is first order with respect to both ferricyanide and oxygen concentration.

#### Energy Considerations

The energy of the light emitted by the DPD's during chemiluminescence is another source of information about the mechanism. Luminol and all the DPD's emit radiation consisting of broad bands with the maxima of the intensities at about 4000 Å (Bremer, 1953). This corresponds to about 60 kcal per einstein. The radiation emitted includes some shorter wavelengths that correspond to 75 or more kcal per einstein. A valid mechanism for the reaction must provide a way for getting this sort of energy into the excited state of a molecule and certainly the reaction must evolve energy of at least this magnitude. However, inasmuch as visible light can be produced from hydrazine and ferricyanide, it is probable that there will be sufficient energy available from the oxidation to form nitrogen of the DPD's to produce the observed light.



their effect on fluorescence yields. Groups like the amino group which greatly increase the intensity of the chemiluminescence generally increase the fluorescence yields of substituted aromatic compounds as can be seen in Table II. This taken with the investigations of Zellner and Dougherty (1937) seems to indicate that the influence of the substituents on the aromatic nucleus is on  $\phi$  rather than on the reaction itself.

TABLE II

Substituent on DPD	Fluorescence, Yield, <sup>a</sup> % (in acid)	Chemilu- minescent <sup>a</sup> Intensity	Chemilu- minescent Fluor. Yields
5—OH	2	20	10
5—Br	2	1	.5
6—NH <sub>2</sub>	2	4	2
5—NH <sub>2</sub>	10	100	10
5—NH·NH·SO <sub>3</sub> Na	0.02	0.2	10
5—NH·COCH <sub>3</sub>	7	1	0.14
5—NH·CH <sub>3</sub>	10	60	6
5—NH·CO·NH·COCH <sub>3</sub>	0.1	2	20

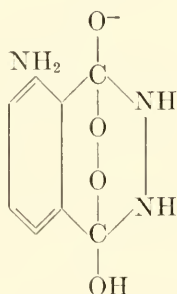
<sup>a</sup> These values are from Spruit (1950).

Hydrazine is weakly luminous when oxidized by ferricyanide. However, the mechanism seems to be different from that involved in the usual chemiluminescence of the DPD's in that oxygen apparently has no effect on the quantity of light emitted. The relatively high efficiency of the light produced by the DPD's appears to require the cyclic ring structure with the two hydrogens available to form the dienol form.

### End Products and Intermediates

The identification of the intermediates and end products of a reaction is of great value in elucidating the mechanism. The efforts to isolate these from the chemiluminescent reaction of the DPD's have not been particularly successful. The conditions required for the chemiluminescence favor the subsequent reaction of the end product and the various substituted phthalic acids that have been isolated, e.g., 3-aminophthalic acid from luminol, might represent ultimate reaction products rather than the direct product of the chemilumines-

cent reaction. The compound 2,2'-dicarboxylbenzil has been obtained by Zellner and Dougherty (1937) and confirmed by Drew and Garwood (1938) from the oxidation of unsubstituted DPD. The fact that this compound can be further oxidized to phthalic acid without the production of light makes it probable that it is more closely related to the actual product of the light producing reaction than the phthalic acid derivatives. The alternative is that it is produced by a side reaction in which case there would be no relationship between it and the product of the chemiluminescence. The preparation of possible intermediates has been reported (Kautsky and Kaiser, 1950; Drew and Garwood, 1938). The compound prepared by Kautsky and Kaiser has not been proven to be related to the chemiluminescence of the DPD's. Drew and Garwood (1938) prepared a compound by dissolving luminol in strong alkali and adding a large quantity of hydrogen peroxide which they assumed to be an intermediate in the chemiluminescent reaction. The subsequent adding of alcohol and cooling caused the precipitation of a crystalline compound. The analysis of this compound showed it to be the mono sodium salt. Drew supposed the compound was the hydrated form of



and did find that one molecule of water could be removed by drying in a vacuum over phosphoric oxide. The subsequent analysis was in substantial agreement with the proposed compound. However, it was found that some hydrogen peroxide was lost by heating and essentially all of it was lost when the compound was heated to 120° C. This seems to indicate that the compound isolated was one where hydrogen peroxide had replaced one molecule of water of crystallization. Drew has reported that the compound dissociated in water and that essen-

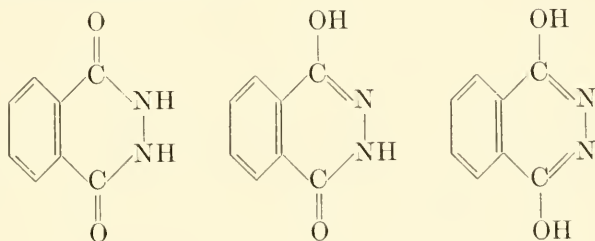


tially all the hydrogen peroxide could be titrated with potassium iodide and dilute sulfuric acid with ammonium molybdate as a catalyst. This would also be characteristic of the compound with hydrogen peroxide replacing water of crystallization. The compound containing the hydrogen peroxide in this form would have the same percentage composition as the one isolated. The evidence therefore seems to indicate that the compound isolated by Drew is not an intermediate in the reaction but a coordination complex with hydrogen peroxide.

Further information is needed concerning intermediates and end products before the mechanism can be fully established. However, a knowledge of the general behavior of molecules makes possible a rather plausible supposition as to the mechanism for this reaction.

### Proposed Mechanism

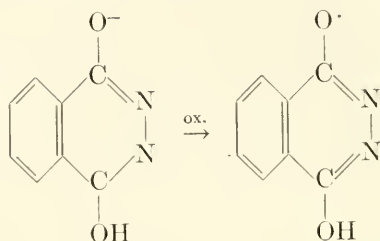
The DPD's probably exist in solution as a mixture of the diketo, mono keto, and dienol forms:



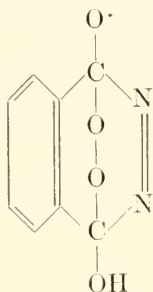
However, in basic solution, the enol forms probably ionize. This would serve to shift the equilibrium in favor of the enol forms. Stross and Branch (1939) have reported a first ionization constant of  $10^{-6}$  for luminol. They have also reported that there was no second ionization constant greater than  $10^{-13}$ . It seems reasonable to suppose that in basic solutions such as are required for chemiluminescence, the major portion of the luminol is in the form of the singly ionized enolic form. In this connection it might be noted that the mono sodium salt is the one normally isolated from sodium hydroxide solutions.

As has been explained earlier, there is evidence that indicates that preliminary oxidation takes place before the reaction with molecular oxygen. Apparently this oxidation can be carried out by any

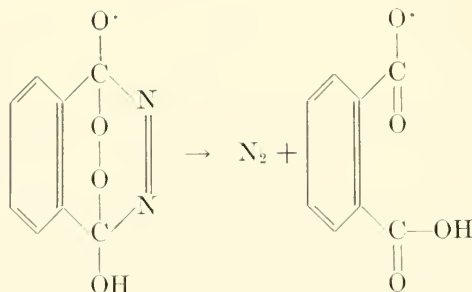
number of oxidants. It might well just involve the loss of the charge on the ionized portion of the molecule



Oxygen then adds to form some such compound as:



This could yield:



The final molecule  $A^*$  can be reduced to a phthalic acid derivative. It can also engage in other reactions, some of which might give rise to the 2,2'-dicarboxybenzil that was isolated by Zellner and Dougherty (1937). The excited molecule  $A^*$  would emit light and undergo the reactions described in equations 3, 4, and 5. The proposed mechanism is speculative and is meant only to serve as a basis for further studies.

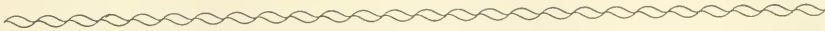
An incidental result of the present study is the demonstration that

the chemiluminescent reaction of luminol and molecular oxygen can be employed as a means of measuring oxygen concentration. The intensity of the light emitted is a function of the amount of oxygen dissolved. The amount of oxygen dissolved can be related to the partial pressure of oxygen in the gas being analyzed. This procedure can be used to measure continuously the concentration of oxygen in flowing systems over reasonable periods of time as the luminol is only slowly consumed. The reaction of luminol with oxygen and an additional oxidant such as ferricyanide should be adaptable to detecting extremely small quantities of oxygen.

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## Spectroscopic Investigations of Luminescent Systems

C. J. P. SPRUIT and A. SPRUIT-VAN DER BURG  
Landbouwhogeschool, Wageningen, Holland

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The present discussion stems largely from the work carried out by the Biophysical Research Group at Utrecht during the period 1935–1947. At the organization of this group, the subject of bioluminescence was chosen by the directors, Prof. Dr. A. J. Kluyver and the late Prof. Dr. L. S. Ornstein, as a promising field for the application of physical methods to biology. During the preceding period, important conclusions had been drawn from the study of the spectroscopic properties of atoms and simple molecules. It was therefore considered advisable to make use of the experience which had been obtained in the Physical Laboratory at Utrecht with methods of spectral energy measurements, for the study of the emission spectra of bioluminescent phenomena.

It was hoped that a detailed knowledge of the spectral energy distribution of bioluminescent emission might lead to conclusions as to the architecture of the molecules involved. It is true that during the following years, the increasing insight into the physics of light absorption and emission by more complicated molecules has considerably tempered this initial optimism.

The theoretical difficulties encountered during the attempt to formulate the connection between chemical structure and spectroscopic properties of organic molecules have been overcome only in some relatively simple cases even now. To these theoretical difficulties should be added the fact that in the case of organic molecules in solution, the ultraviolet and visible absorption spectra are of a diffuse

nature, consisting of broad bands in which vibrational and rotational fine structure is almost always lacking. Moreover, in the case of emission spectra such as fluorescence spectra, the observed emission band corresponds to only one electronic transition in the molecule so that such spectra are even less detailed than the absorption spectra. This does not take away the fact that in the study of bioluminescence, the emission spectra are the only data obtainable that are immediately and uniquely connected with the bioluminescent compounds. In this connection, an accurate knowledge of spectra should be highly desirable.

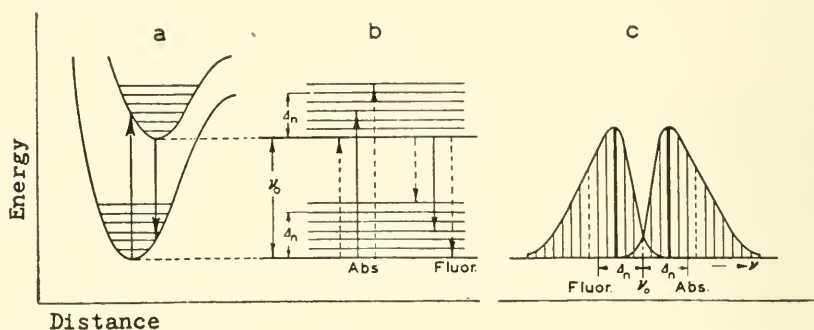


FIG. 1. a and b. Energy states for a two-atom molecule with transitions in absorption and fluorescence. c. The occurrence of mirror symmetry between absorption and fluorescence spectrum.

It needs no emphasis that bioluminescence is a form of chemiluminescence. The known chemiluminescent systems offer a means of studying the relation between the chemiluminescence emission spectrum and other spectral properties of the compounds involved. During the chemiluminescent reaction, molecules are transferred to an excited state, and this is followed by the emission of light. Therefore, the process of light emission during a chemiluminescent reaction is related to the light emission during fluorescence. It is therefore desirable to say a few words at this point about the nature of fluorescence and its relation to light absorption in organic molecules. We will consider the case of a two-atom molecule. In Fig. 1a, two electronic energy states of this molecule are given, together with some vibrational levels. The arrows indicate a transition from the ground level to a higher



energy level by absorption, and from the higher to the ground level by the emission of light. The same is represented in another way in Fig. 1b. As long as a number of conditions are fulfilled as to transitional probabilities and spacing of vibrational levels in the two energy states, it may be predicted that there is a mirror symmetry between the emission and the fluorescence spectrum, if plotted versus frequency, as shown in Fig. 1c.

The reemission of light by the excited molecule is not the only way

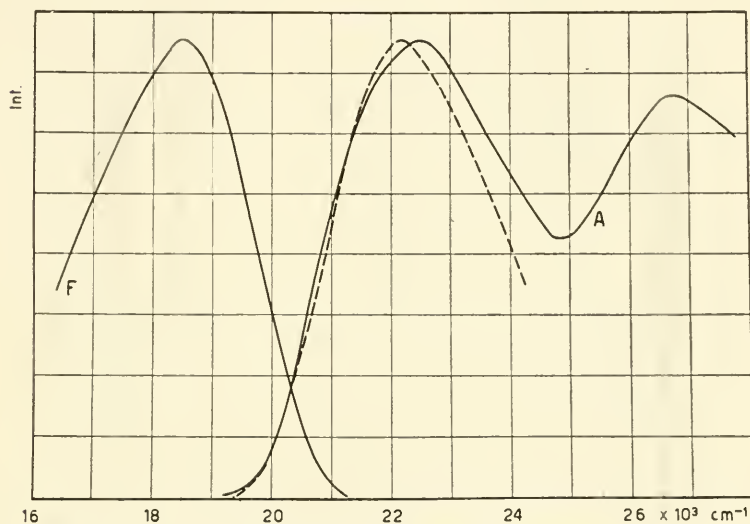


FIG. 2. Mirror symmetry as observed in practice. Fluorescence (F) and absorption (A) spectra of riboflavin plotted against frequency. Dash line: mirror image of fluorescence spectrum.

by which this energy can be dissipated, and, as a rule, the molecule returns to the ground level by way of other transitions not involving the emission of a quantum. In this case the energy is lost in the form of heat. Only if special conditions are fulfilled are such radiationless transitions of sufficiently low probability that fluorescence can occur with a measurable quantum yield, and only in relatively rare cases does this yield approach unity. It is an empirical fact that only for the first excited level are the probabilities of these radiationless transitions to the ground level sufficiently low to allow for the occurrence of fluorescence. For this reason, only those emission bands are en-

countered that correspond to the long-wavelength absorption band in the absorption spectrum. In Figs. 2 and 3, examples are given of this mirror symmetry between absorption and fluorescence for some compounds of interest in connection with the following discussion. For a discussion of fluorescence of organic compounds the reader is referred to Förster (1951).

Returning to the chemiluminescence spectra, the question arises as to the mechanism of excitation. We may visualize two possibilities:

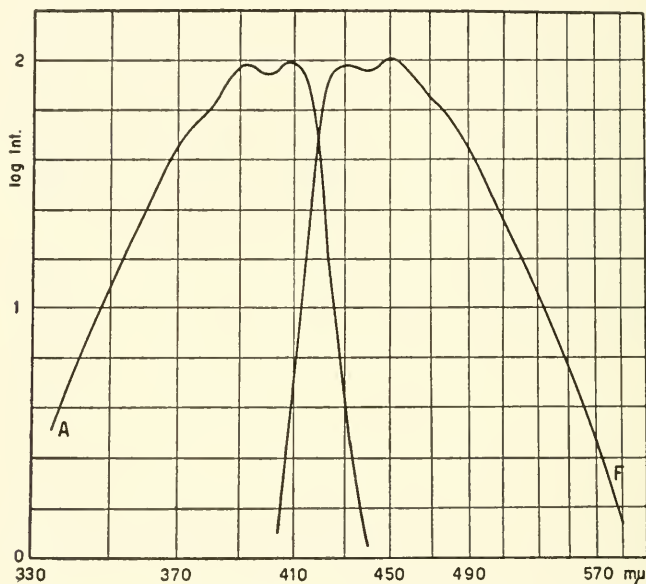


FIG. 3. Mirror symmetry in the fluorescence and absorption spectra of methylacridone.

during the reaction, intermediates or end products are directly formed in an excited state, or they may obtain their energy by transfer from other reactants containing sufficient energy. It is not easy to distinguish experimentally between these possibilities.

Kautsky (1943) is believed to have shown that the emission spectra of various chemiluminescent reactions were very similar to the fluorescence spectra of the parent compounds. This has led many investigators to the assumption that during the chemiluminescent reaction, the original molecules are regenerated, but in an electronically excited

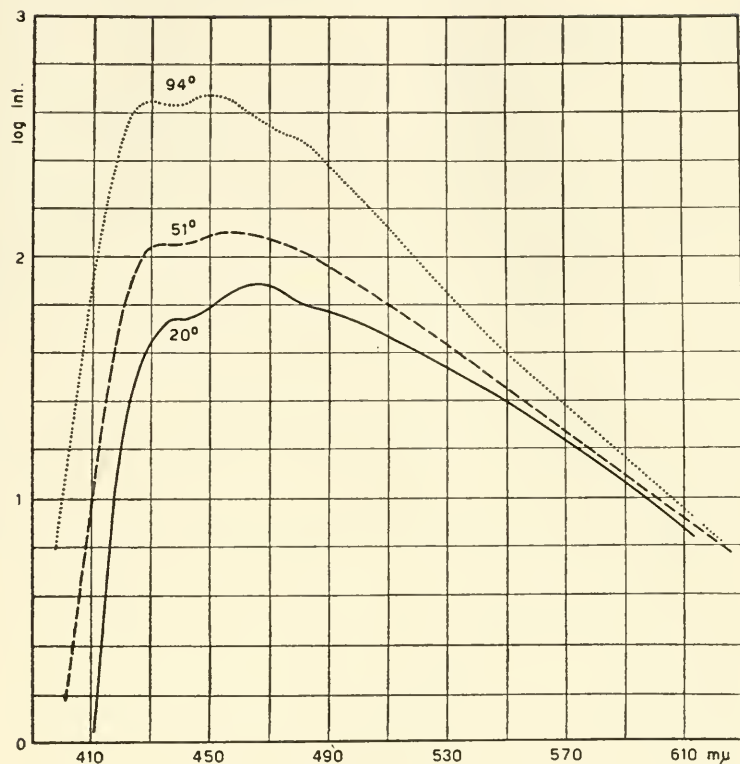


FIG. 4. The emission spectra of the chemiluminescence of dimethylbisacridinium nitrate at three temperatures.

state. Though there is as yet no experimental evidence ruling out this possibility, there is no sound basis for the assumption, as we shall see.

Transfer of excitation energy, although theoretically possible, has not been observed with certainty during chemiluminescence in solution. In this connection it is advisable, however, to recall the old observations by Kautsky (1925), who observed chemiluminescence of organic dyes adsorbed upon inorganic gels, where the excitation energy obviously originated from oxidation of the adsorbent. This work certainly merits reconsideration and reexamination with modern methods. It has been demonstrated by Duysens (1952) that transfer of excitation energy between organic pigments in the living plant cell is a normal phenomenon. It is therefore advisable to keep in mind

this possibility when considering the nature of the light-emitting molecules in bioluminescence.

At Utrecht, Miss van der Burg (1943, 1950 a and b) examined the emission spectra of a number of chemiluminescent reactions in solution and the fluorescence spectra of the chemiluminescent compounds. In Fig. 4, the emission spectra are given of the reaction of dimethylbisacridinium nitrate with hydrogen peroxide at three different temperatures. Obviously, in this case there is more than one chemiluminescent reaction, and at temperatures between 20° and 94° C, the resulting emission spectra could be described as linear combinations of two different spectra, I and II. The spectrum obtained at 94° C is practically identical with I, and the low temperature spectrum is very close to II. The general form of these two spectra makes it likely that both belong to derivatives of acridine, e.g., Fig. 3. Now it appears that the high-temperature component, I, is very similar to the fluorescence spectrum of methylacridone, Fig. 5. The two spectra are not

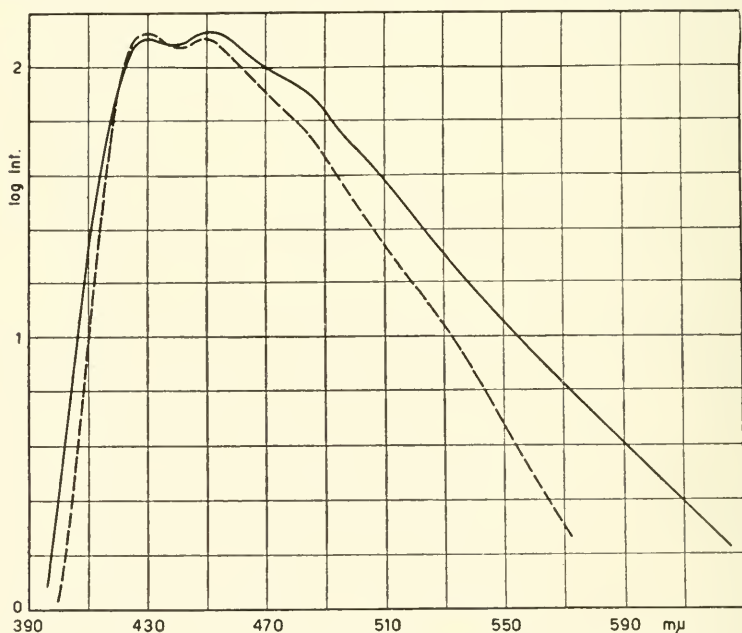


FIG. 5. A comparison of the high-temperature chemiluminescence spectrum of dimethylbisacridinium nitrate (—) with the fluorescence spectrum of methylacridone (---).

identical, however, and the difference is well outside the limits of experimental error. It is nevertheless tempting to identify at least one of the emitting molecular species with the main end product of the chemiluminescent reaction, methylacridone. As the fluorescence spectrum of this compound, recorded in Fig. 5, has been measured at room temperature, it is not impossible that the higher temperature during the reaction is responsible for this broadening. An examination of the absorption spectrum of methylacridone at 94° C indeed shows such a broadening in the absorption, but the effect does not

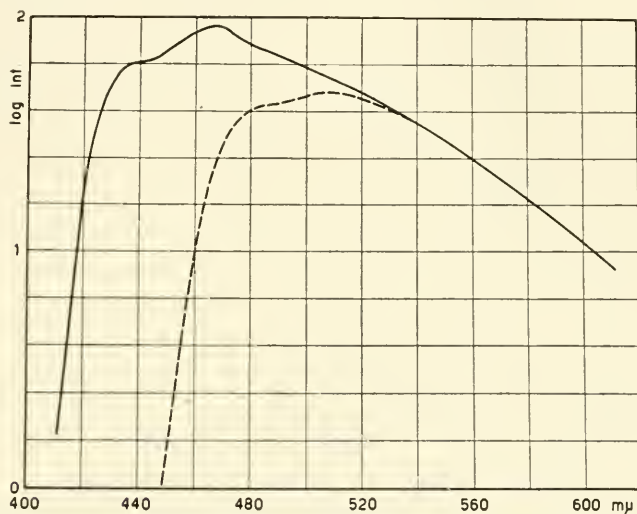


FIG. 6. A comparison of the low-temperature chemiluminescence spectrum of dimethylbisacridinium nitrate (—) with the fluorescence spectrum of this compound (---).

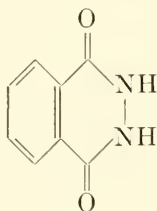
seem to be sufficient to explain the broadening of the high-temperature chemiluminescence spectrum.

The nature of the second emitting molecule is not known. It certainly is not dimethylbisacridinium nitrate, as its spectrum extends much more to the violet than does the fluorescence spectrum of that compound, Fig. 6.

At this point it appears advisable to point out some sources of confusion to those examining emission spectra of colored solutions. As a rule, the emission spectra, such as fluorescence and chemiluminescence

spectra, overlap more or less with the absorption spectra. As it is advantageous to have as high a light intensity as possible for the study of the emission spectra, it is tempting to use highly concentrated solutions. This, however, has the result that in the case of fluorescence practically the whole short-wavelength part of the emission spectrum may be lost by self-absorption. In chemiluminescence, this overlap may be even greater. Moreover, if the fluorescence yield of the solution is not very low, the absorbed radiation is reemitted for an important part as fluorescence. This means that in the examination of fluorescence spectra the short-wavelength part of the emission spectrum will be suppressed, and in the examination of chemiluminescence spectra one may arrive at the erroneous conclusion that the emission spectrum is identical with the fluorescence spectrum of the starting material. In this way, Kautsky (1943), who first studied the chemiluminescence spectrum of dimethylbisacridinium nitrate, may have arrived at his conclusion that at low temperatures the emission spectrum is identical with the fluorescence spectrum. This conclusion certainly is erroneous as is demonstrated by Fig. 6. In the experiments of van der Burg, special attention has been paid to this source of error, and it can be stated that the fluorescence of dimethylbisacridinium nitrate certainly contributes less than 3% to the spectrum of Fig. 6. To avoid errors as those discussed above, it is therefore necessary to use relatively dilute solutions and to apply a correction to the emission spectrum for the absorption by the solution.

In the case of the chemiluminescence of the derivatives of 2,3-dihydrophthalazinedione (DPD)



the results were less clear-cut. We will discuss the most illustrative examples, namely the spectra of 5-amino-DPD and 5-methylamino-DPD, Fig. 7. It was found as a general rule that, as far as the wavelengths of maximum emission are concerned, the chemiluminescence



spectra of these compounds are similar to the fluorescence spectra in acid medium, but different from the fluorescence spectra in alkaline media, in which the luminescent reaction is carried out. It may be added that for practically all compounds investigated, the fluorescence

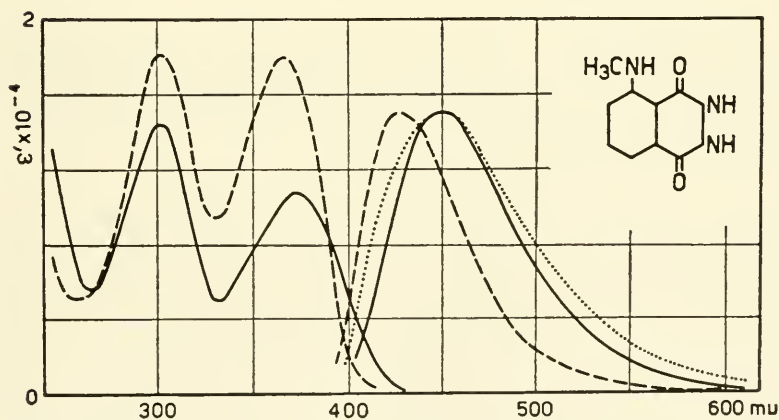
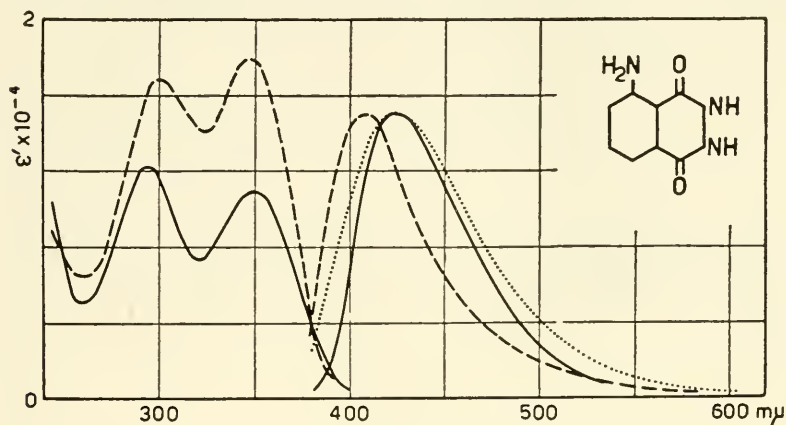
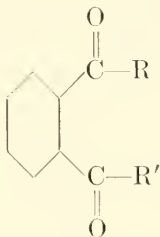


FIG. 7. The absorption, fluorescence and chemiluminescence spectra of two derivatives of dihydrophthalazinedione. — Absorption and fluorescence spectra of acid solutions. - - - Absorption and fluorescence spectra of solutions in 0.01 N KOH. . . . Chemiluminescence spectra.

yield under the conditions of the reaction is practically nil. Notwithstanding the similarity, the chemiluminescence emission spectra are generally broadened with respect to the fluorescence spectra, just as in the case of the high-temperature spectrum of dimethylbisacridinium nitrate. If this broadening of chemiluminescence spectra relative to fluorescence spectra proves to be a general feature, it may be of considerable theoretical interest.

Our only conclusion from the results discussed above is that the chemical structure of the emitting molecule is similar to that of the acid, diketo form of the derivatives of DPD. As the absorption spectra of these compounds are not very characteristic, a further identification is impossible, and in particular it is not possible to decide whether or not the heterocyclic ring is still intact in the emitting molecule. Its structure may be represented therefore by a general formula of the type



The interpretation of chemiluminescence spectra touches upon the question of the reaction mechanisms and the occurrence of intermediate products. Attractive as this aspect may be, it is surprising that so little is known about it with certainty.

We will now turn to the examination of the emission spectra of luminous organisms. From the foregoing we may expect them to be related to fluorescence spectra of reaction products of the molecules entering into the luminescent reaction. Hence they should show vibrational structure only in so far as the fluorescence spectra and in particular also the longest-wavelength bands in the absorption spectra of the compounds involved show such vibrational structure. The occurrence of secondary maxima, such as those found by Eymers and van Schouwenburg (1937), in emission spectra of various types should be of special interest. We regret to say that more careful measurements have failed to confirm the claims of these authors.

In making spectral energy measurements by the photographic method, one should always take averages of a considerable number of separate exposures, as the plates are never quite homogeneous. Other points to be observed in the measurement of emission spectra consisting of diffuse bands are absorption and scattering. In this case it is not possible to apply a correction for these effects to the observed emission spectrum, and the only solution is therefore to make use of such dilute suspensions that they may be neglected. The absorption

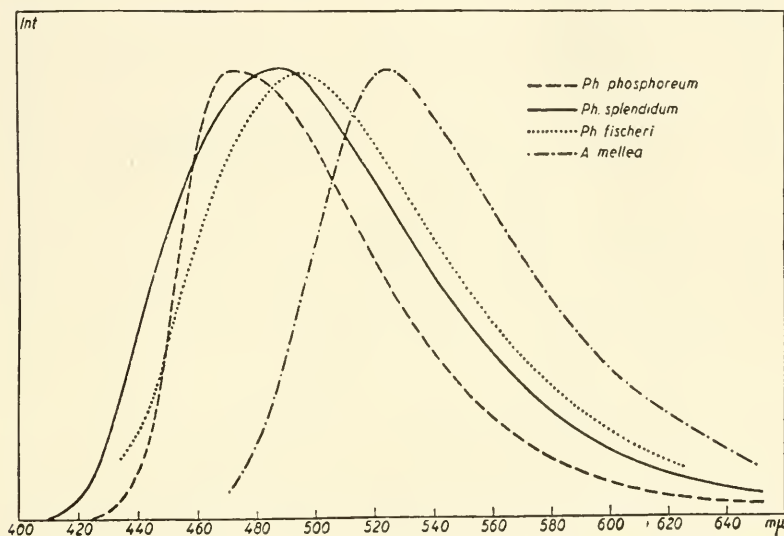


FIG. 8. Bioluminescence emission spectra of three species of luminous bacteria and one fungus. (From *Bioch. et Biophys. Acta* 5 (1950), by permission of Elsevier Publishing Company.)

of the emitted light by the emitting cell itself is unknown, however, and the spectra may be still in error by this amount. In the emission of luminous bacteria, a calculation shows that in all probability this absorption by only one cell is negligible. In luminous fungi, however, one is compelled to make use of mycelial mats, consisting of several layers of hyphae. Here absorption may not be without importance.

The spectra obtained by van der Burg (1950c) for various organisms are reproduced in Figs. 8 and 9. What conclusions can be drawn from these spectra? In the first place, it is likely that the three fungi have identical emission spectra and hence identical bioluminescent

systems. These appear to differ from those of the three bacteria. As regards the latter, *Ph. phosphoreum* certainly deviates much from the others. Although *Ph. splendidum* and *Ph. fischeri* have different spectra, it is perhaps not advisable to take their difference for granted as we have to account for the absorption of the light by the bacteria themselves. Although this is probably negligible, *Ph. fischeri* has a distinctly yellow color and in this case the absorption may well be of some importance. This should affect the short-wavelength part of the spectrum in particular.

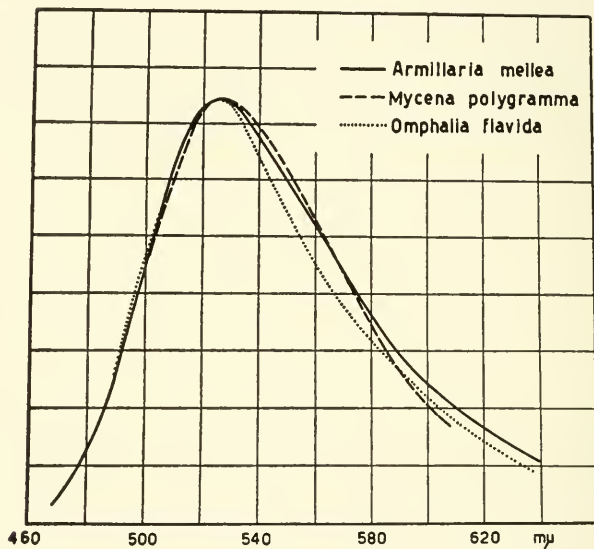


FIG. 9. Emission spectra of three luminescent fungi.

Occasionally spectra of bioluminescent processes have been published. It is difficult to compare them with those just discussed, as it is uncertain and even unlikely that the precautions we have mentioned have been observed during their measurement. For example, two spectra of the emission of *Cypridina* have come to our knowledge, one by Coblenz (1926), the other by Eymers and van Schouwenburg (1937), showing maximum emission at about 480 and 464 mμ respectively. Both spectra have been obtained by moistening *Cypridina* powder before the slit of the spectrograph. This powder is a strongly

colored substance with considerable absorption in the blue and violet regions of the spectrum. From visual observation we feel reasonably sure that the emission maximum of *Cypridina* is at shorter wavelengths than that of *Ph. phosphoreum* ( $472\text{m}\mu$ ). Three spectra of the emission of *Photinus pyralis* are known to us. They do not agree among each other save that the maxima of emission are rather close together ( $563$ ,  $565$ , and  $568\text{ m}\mu$ ). As this is far in the green, where there is not much self-absorption, this maximum should be quite reliable.

At any rate we may conclude that the emission spectra do prove that the emitting molecules in these various organisms are definitely different. As all these spectra are related to one band in the absorption spectra of the compounds involved, it will be clear that little information can be drawn from them without the assistance of further independent observations.

It has been suggested on several occasions that bioluminescence is connected in some way or other with the presence in the cell of derivatives of riboflavin. It is therefore advisable to point out that none of the emission spectra obtained so far show much resemblance with the fluorescence of riboflavin (Fig. 2), the maximum of which is situated at  $534\text{ m}\mu$ .

If the luminescent system occurs in the cell in the form of an enzyme with a specific substrate, the concentration of this compound in all probability will be so low that direct observation by absorption spectroscopy will be impossible. Accidentally, during a study of carbon monoxide inhibition of luminescence, van Schouwenburg and van der Burg came across an indirect method for the measurement of the absorption spectrum of the luminescent system in *Ph. phosphoreum*. The idea has been the basis of an investigation by van der Kerk and van der Burg. Whereas irradiation with red light has no effect upon luminous bacteria, irradiation with comparable intensities of blue light tends to dim the light emission of the bacteria. A quantitative study of this effect, which is fully described in the thesis of van der Kerk (1942) (Kluyver *et al.*, 1942) led to the establishment of an action spectrum for the quenching of bacterial luminescence which should be proportional to the absorption spectrum of the system which emits the light. Later observations (Spruit, 1946, 1949a) have necessitated a correction to be applied to this spectrum at wavelengths below

300  $m\mu$ . The corrected spectrum is given in Fig. 10. Unfortunately, this spectrum is not very detailed, and it is not immediately obvious to what class of compounds it has to be attributed. The only safe conclusion to be drawn from it is that it does not belong to any of the well-known, generally occurring cellular components such as caro-

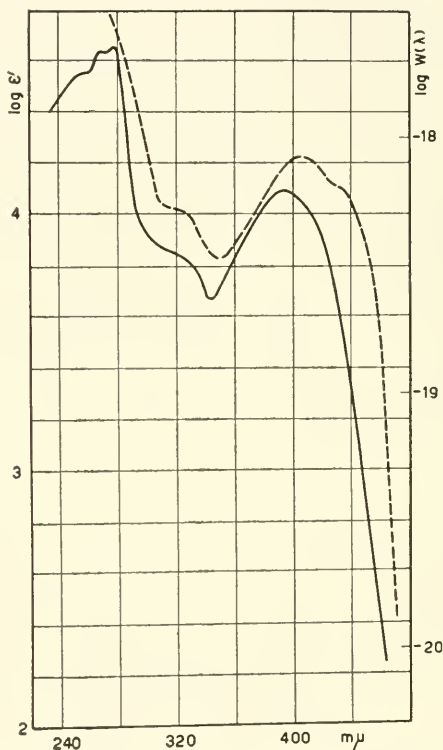


FIG. 10. The photochemical inactivation spectrum of *Ph. phosphoreum* (---) and the absorption spectrum of 1,4-dihydroxynaphthyl-2-hydroxymethylketone (—).

tenoids, flavine enzymes, hematin compounds, or to systems containing any of the colored vitamins. This supports van der Kerk's conclusion that it really represents the spectrum of a system, closely connected to the bioluminescent reaction and not merely the spectrum of a sensitizer.

Another important piece of information lies in the recovery of lumi-



nescence after the end of the irradiation, which is a first order reaction. This recovery of luminescence also exists in those bacteria which have lost their reproductive capacity by exposure to wavelengths shorter than 300 m $\mu$ . Ultimately, such "killed" bacteria emit light many times more intense than a nonirradiated part of the same culture. This observation is in perfect harmony with the old views of van Schouwenburg that bacterial luciferin has a dual function and that part of it is involved in a reaction other than the luminescent one. Obviously in such damaged bacteria more luciferin is accessible to the luminescent reaction after recovery is complete than in normal bacteria. This may well be due to the fact that that part of the respiratory system responsible for the reversible dehydrogenation of luciferin (see van Schouwenburg, 1938) is inactivated by irradiation with light of short wavelengths. Although definite proof is lacking, these facts support the conclusion that van der Kerk's inactivation spectrum indeed is the absorption spectrum of the bacterial luciferin or its immediate precursors.

The same authors also made a few observations on the inactivation spectrum of *Ph. splendidum* and *Ph. fischeri*, though only in the visible region. It is highly regrettable that they have not found it possible to include in their study the ultraviolet parts of these spectra, but from what has been published it is clear that, whereas the inactivation spectra of *Ph. splendidum* and *Ph. fischeri* cannot be distinguished on the basis of the material available, they are certainly not identical with the inactivation spectrum of *Ph. phosphoreum*. The situation is therefore somewhat similar to that of the emission spectra. This observation raises the question of mirror symmetry. As was explained earlier, chemiluminescence emission spectra, being related to fluorescence spectra, should show qualitative mirror symmetry with the long-wavelength bands in the absorption spectra of the compounds in question. If we observe a certain amount of symmetry between the emission and the inactivation spectra of a number of organisms, we may therefore take this as a fair indication that both types of spectra belong to the same, or at least to pairs of closely related compounds. Now, this is what is really observed, Fig. 11. The form of the inactivation spectra really is rather different, but the important point is the small overlap for all pairs of spectra. This comparison therefore is a

material support for the attempt to attribute both the emission and inactivation spectrum to the same system.

Although the information that can be obtained from the detailed form of the photochemical inactivation spectrum of *Ph. phosphoreum* is not great, it has more weight if the additional information we have about the chemical nature of bacterial luciferin is taken into account. As van Schouwenburg has amply discussed (1938), bacterial luciferin

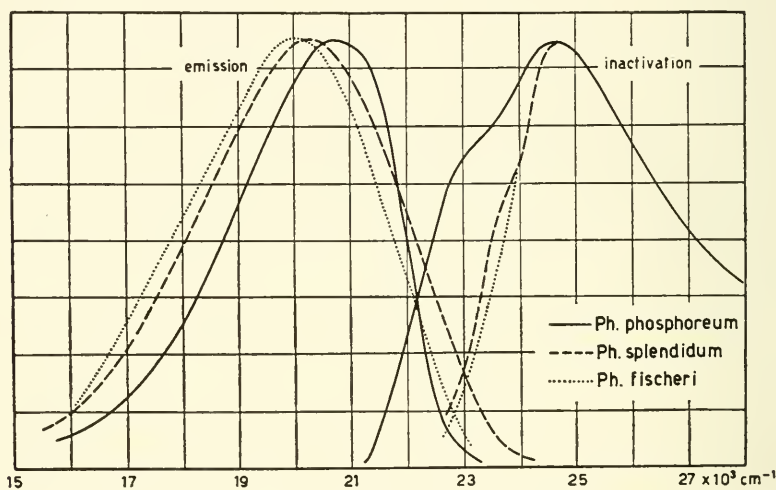
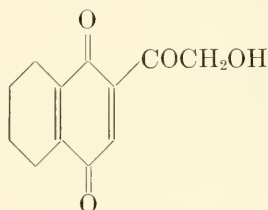


FIG. 11. Qualitative mirror symmetry between the emission and photochemical inactivation spectra of three luminous bacteria.

may be reversibly oxidized and reduced in the bacterium, just as was demonstrated previously by Harvey for *Cypridina* luciferin. At the time the inactivation spectrum was measured, we still believed it a reasonable assumption that the various luminescent systems found in nature were at least related. It is probably for this reason that we have thought it permissible to take into account the results of observations with other organisms in making speculations about the chemical nature of the compound whose inactivation spectrum had been measured.

One of the very few bits of American literature which came to us during World War II was a publication by Chakravorty and Ballen-

tine (1941), which contained results of the resynthesis of luciferin from its irreversible luminescent oxidation product. We were not in a position to check the results reported and we may have attached too much value to them. Taking all information together, van der Kerk suggested that the compound whose absorption spectrum is the inactivation spectrum had as part of its structure:

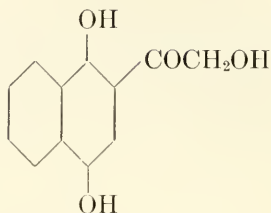


From available data on the absorption spectra of naphthoquinones (Macbeth *et al.*, 1935) the assignment of a naphthoquinone structure to the compound appeared justifiable.

Although the situation at the moment no longer makes it especially attractive to look for a relationship between the various forms of bioluminescence, at that time it appeared promising to test this hypothesis further by a study of the absorption spectra of naphthoquinones, and in this connection an attempt was also made to prepare the compound mentioned by van der Kerk. Accepting his idea as a working hypothesis, it appeared necessary to establish with more certainty the state of reduction of the hypothetical compound. According to van der Kerk, this should be the quinone form. This conclusion he based mainly on the observation that several quinones (e.g., 2-methylnaphthoquinone) are known to be photolabile. A closer analysis of the data, especially van der Kerk's experiments on the photo-inactivation under low oxygen pressure, have convinced us that it is much more likely that the reduced form of luciferin is the photolabile component (Spruit, 1949a). This is in harmony with the observation that 1,3,4-trihydroxynaphthyl-2-methylketone, a derivative of naphthohydroquinone, is also photosensitive in the presence of low pressures of oxygen (Spruit, 1947). It was therefore quite an exciting observation that the absorption spectrum of 1,4-dihydroxynaphthyl-2-hydroxy-



methylketone, the hydroquinone of van der Kerk's hypothetical compound



is very similar to the corrected inactivation spectrum, Fig. 10.

These observations were made in 1947. We immediately tested the compound with luminous bacteria and with *Cypridina* luciferase (Spruit, 1949a), but without success. In view of the number of hypotheses upon which our attempt was based, this was not very surprising, and as there is no immediate way of further testing the idea, this is where our story could come to an end. However, during the years that followed, new contributions were made by other workers to the subject of bioluminescence, and apart from van der Kerk's inactivation spectrum we have now two more spectra of compounds involved in bioluminescent reactions. One is the absorption spectrum of purified *Cypridina* luciferin, measured by Chase and Brigham (1951); the other is that of the stimulating factor in the luminescence of the firefly *Photinus pyralis*, measured by Strehler and McElroy (1949). A comparison of these spectra serves to extinguish our last hope that the various forms of bioluminescence might be related. It is certain that these three spectra belong to widely different classes of compounds. We also have to conclude that most of the arguments in favor of van der Kerk's proposal are of doubtful value, and the nature of the compound involved in the photochemical inactivation is therefore still unknown.

Notwithstanding these criticisms, the remarkable similarity between the inactivation spectrum of the bacteria and the absorption spectra of certain derivatives of naphthohydroquinone is striking. It is hard to believe that this similarity is completely meaningless. We must recall here also the magnificent work by van Schouwenburg (1938), which has given a firm basis to the theory that in the bacterium *Ph. phosphoreum* the luciferin is reversibly oxidizable and reducible, a

conclusion that has received considerable support from the work of Spruit and Schuiling (1945). So we know at least one chemical property of this compound which is in agreement with the chemical structure of the naphthoquinones.

It may be argued that although certain derivatives of naphthohydroquinone possess absorption spectra quite similar to van der Kerk's inactivation spectrum, it might well be that other unrelated classes of compounds exist with similar spectral properties. During later years we have had opportunity to study the ultraviolet absorption spectra of various compounds in more detail, and the results of this work make it appear justifiable to reconsider the matter of an identification of the bacterial luciferin. We should like to present some of the relevant material here.

How typically characteristic is the absorption spectrum mentioned of this particular class of compounds? To test this, one can investigate the influence upon the absorption spectrum of alterations in the substituents attached to the naphthalene nucleus. The second method is to change the character of the aromatic nucleus itself. If we take naphthyl-2-methylketones as a starting point, it is clear from theoretical considerations that substitution in the methyl group should not alter the absorption spectrum profoundly. This is confirmed by the experiments (Spruit, 1949c). On the other hand, keeping in mind that the long-wavelength absorption band in the inactivation spectrum is somewhat more to the red than the corresponding band in the naphthohydroquinone compound, we are interested in the effects of other substituents in the nucleus. Figure 12 gives examples of what happens if one shifts around with the OH groups. The result demonstrates that the exact position of the long-wavelength band should not pose much of a problem. Further examples can be found elsewhere (Spruit, 1949b).

Quite another problem is posed by the nature of the aromatic nucleus. As far as the quinones are concerned, the absorption spectra of anthraquinones are very similar to or almost identical with those of the analogous naphthoquinones. The quinone nucleus forms an effective barrier between the conjugated systems on both sides of it. This observation is amply confirmed by the spectra of a number of other tricyclic paraquinones containing heterocyclic ring systems (Spruit,



unpublished). On the other hand, the tricyclic hydroquinones show a type of absorption spectrum, quite different from those of the naphthohydroquinones.

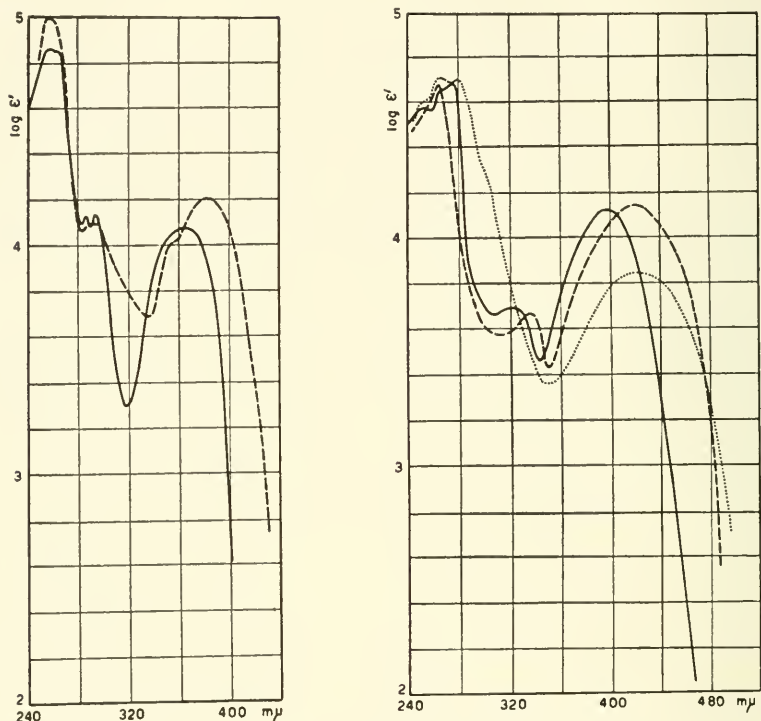


FIG. 12. The effect of substituents upon the absorption spectra of naphthyl-2-methylketones. Left: — 1-hydroxynaphthyl-2-methylketone, - - - 1,5-dihydroxynaphthyl-2-methylketone. Right: — 1,4-dihydroxynaphthyl-2-methylketone, - - - 1,5,8-trihydroxynaphthyl-2-methylketone, . . . 1,3,4-trihydroxynaphthyl-2-methylketone.

In how far are the absorption spectra determined by the properties of the aromatic ring system? To answer this question one should like to have a means of continuously altering these properties. Obviously this is impossible, and the next best one can do is to compare a series of analogous quinones containing different heterocyclic ring systems, instead of the naphthalene nucleus. If the hetero atoms show different electron affinities, we have more or less what we want.



Accordingly we have examined paraquinones derived from benzofuran, thianaphthene, selenanaphthene, and isoindazole.

In the first place it was found that the properties of the pyrazole

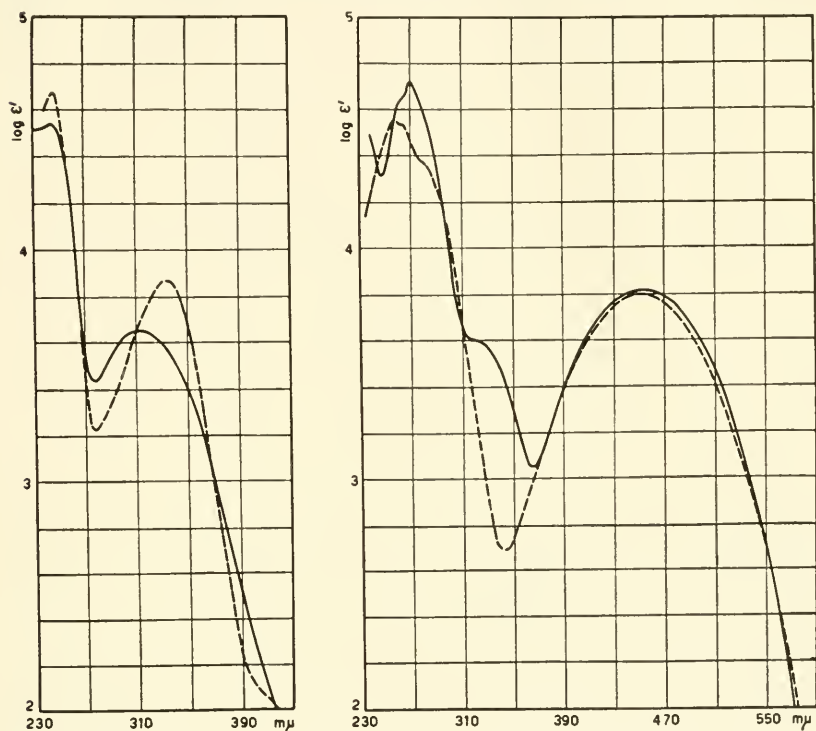
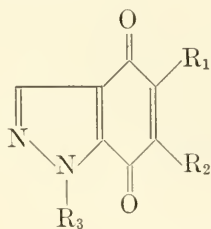
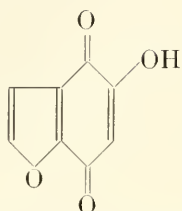


FIG. 13. Alterations of the nucleus. Similarity between the absorption spectra of isoindazolequinones and of analogous naphthoquinones. Left: — isoindazole-4,7-quinone, - - - - 1,4-naphthoquinone. Right: — alkaline solution of 2-hydroxynaphthoquinone, - - - - alkaline solution of 5-hydroxyisoindazolequinone.

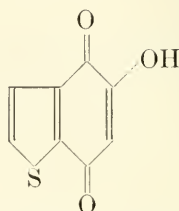
nucleus are very close to those of the benzene ring, as the absorption spectra of the isoindazolequinones



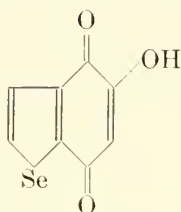
are very similar to those of the analogous naphthoquinones, Fig. 13. On the other hand, if we compare with 2-hydroxynaphthoquinone the series:



5-Hydroxybenzofuranquinone



5-Hydroxythianaphthenequinone



5-Hydroxyselenanaphthenequinone

we have such a series of compounds with decreasing electron affinity of the hetero atom. This leads to interesting changes in the absorption spectra, Fig. 14. The bands marked with *a*, *a'*, and *a''* appear to form a group related to each other and to the short-wavelength bands of the naphthoquinones. The same holds for the groups marked *b* and *c* respectively which compare with the 330-m $\mu$  and long-wavelength bands of the naphthoquinones. The less the electron affinity of the hetero atom, the more "aromatic" is the character of the nucleus and the more are the bands in the absorption spectra shifted toward longer wavelengths. This holds least for the long-wavelength bands but we will not go into the reason for this here. As is well known, furan has only very weak aromatic properties, and this agrees with the observation that the absorption spectrum of 5-hydroxybenzofuranquinone is very similar to that of dihydroxybenzoquinones. So far we have not yet had the opportunity to investigate the absorption spectra of the corresponding hydroquinones in detail, owing to their unstability. There is no reason to expect a deviation from what has been discussed above, however.

This brief summary may suffice to show that it is certainly possible to prepare compounds with spectra duplicating those of the naphthoquinone derivatives. It is at the same time clear that in order to obtain compounds with absorption bands more or less at the same

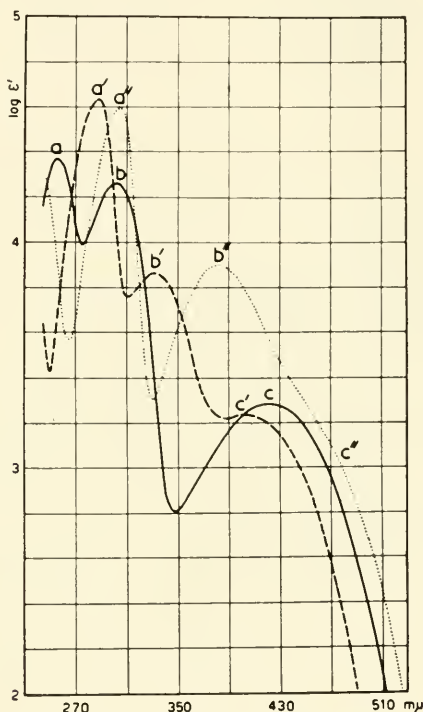
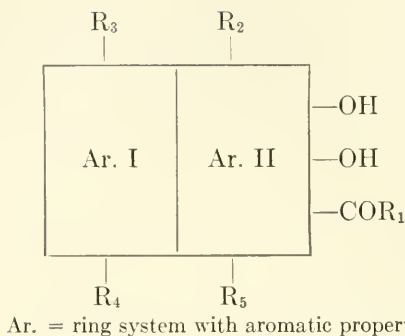


FIG. 14. Alterations of the nucleus: — 5-hydroxybenzofuranquinone, - - - 5-hydroxythianaphthenequinone, . . . 3,6-dimethyl-5-hydroxyselenanaphthenequinone.

position as those in the inactivation spectrum, certain restrictions are imposed as far as the nature of the nucleus is concerned. Its properties should be close to those of naphthalene. The results of the investigations outlined above are therefore certainly not in disagreement with the view that bacterial luciferin has a chemical structure which may be symbolized as follows:



$R_1 \dots R_5$  may represent other groups, not contributing materially to the absorption spectrum, but required in connection with the other functions of the molecule.

If we now attempt to summarize the conclusions that can be drawn from the spectroscopic investigations of bioluminescent phenomena, we are forced to admit that the results have not come up to the original expectations. It is not possible to draw definite conclusions as to the structure of the emitting molecules from the bioluminescent emission spectra, and it is not likely that further theoretical developments will make such a conclusion possible. On the other hand, the divergence between the emission spectra of various organisms is a demonstration that the emitting systems are considerably different. Only in the case of the three fungi examined, has the conclusion to identify the bioluminescent processes seemed justified. At the same time, the study of chemiluminescence spectra has led to the important observation that in this case, emission spectra are closely related to fluorescence spectra of molecules involved in the luminescent reactions. This makes it attractive to compare bioluminescence emission spectra with fluorescence spectra of known biologically important compounds. This comparison does not point to an identity of the emitting molecules with such generally occurring substances. This same conclusion follows from an examination of the three absorption spectra of bioluminescent compounds, obtained so far. Together, these facts seem to indicate that bioluminescence is the result of certain specific and, as yet, not fully known metabolic steps. Moreover, it is likely that different organisms have fundamentally different types of luminescent systems which may not be even superficially related.

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## DISCUSSION

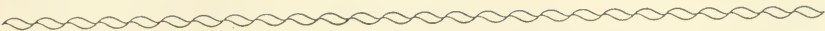
**Dr. Mason:** One of the major purposes of Dr. Spruit's work has evidently been to characterize *Cypridina* luciferin by means of systematic study of the absorption spectra of certain chromophores. Since very small quantities of substance are required for the measurement of absorption spectra, the method has obviously a wide application to the determination of structure of natural products, and it is of interest to consider what its limitations may be.

Aside from technical problems, such as the establishment of homogeneity, the principal limitation lies in the presupposition that it is possible to assign a structure to a light-absorbing molecule with a high degree of confidence when only the absorption spectrum is available. In so far as the ultraviolet and visible regions of the spectrum are concerned, very large classes of compounds tend to absorb light in certain well-defined regions such as 260 to 280 millimicrons. If the substance we wish to characterize displays a single absorption band in this region, the confidence with which we can assign a structure to it is very low indeed, even if the correspondence between the absorption spectra of the known and unknown substances is high. If the unknown substance displays two absorption maxima in the ultraviolet and visible region and the correspondence between the absorption spectra of the known and unknown substances is again relatively exact, the degree of confidence in the structural identification rises correspondingly. Extending this line of thought, *the larger the number of corresponding uniquenesses between the spectra of known and unknown substances, the higher the degree of confidence we may have in the identification.* Characteristic absorption maxima for a single compound can be multiplied by observing spectral changes which can be induced by changes in environment or by frank reaction, that is, alterations in pH, in solvent type, or by autoxidation.

There is one important reservation which should be made in regard to the amount of structural detail which is revealed by absorption spectra in the ultraviolet and visible regions. In order to be spectroscopically detectable, each atom or system of atoms in an absorbing molecule must have the capacity to affect, detectably and characteristically, the levels of excitation to which the molecular orbitals can be raised. Inasmuch as there are very large numbers of possible alter-

ations in chromophoric molecules which will have virtually no detectable effect upon the absorption spectrum of the substances in these regions, the degree of confidence in identification by means of correspondence between electronic spectra must be correspondingly limited.

This general line of argument may be readily extended to identifications derived by correspondences between the spectra of substances in the infrared region.



## Recent Studies on the Chemistry of *Cypridina* Luciferin\*

FREDERICK I. TSUJI, AURIN M. CHASE, and E. NEWTON HARVEY  
Biological Laboratories, Princeton University, Princeton, New Jersey

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### Historical Introduction

The luminescent system in the marine ostracod crustacean *Cypridina hilgendorfi* is one of the simplest among luminous animals. Only luciferin, luciferase, and dissolved oxygen appear to be necessary for light production in aqueous solution. In this respect the *Cypridina* system differs from that of the firefly, where luciferin, luciferase, adenosine triphosphate, magnesium ions and oxygen are all essential (see McElroy and Coulombre, 1952, and Hastings, McElroy and Coulombre, 1953, for latest results).

Structurally, the luminous organ of *Cypridina*, the submaxillary gland, is also simple, made up of elongated gland cells, whose contents are squeezed into the sea water through individual openings on the tip of the upper lip by contraction of muscle fibers. Observation of the living animal indicates that two varieties of granules pour out of the submaxillary gland, one large (diameter about  $10\ \mu$ ) and yellow, undoubtedly luciferin, and another small (diameter about  $2\ \mu$ ) and colorless, probably luciferase. Both types of granules dissolve on meeting the sea water, and at the same time the luminescence appears (see Harvey, 1952, for details of gland structure).

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Whole *Cypridinae* can be dried, and if the drying process is rapid and the material preserved with a drying agent, the ability to luminesce brightly on adding water will last indefinitely (at least 32 years). This dried material has been the starting point for most of the chemical studies on luciferin and luciferase. The early work of Harvey (1917–19) established that the *Cypridina* luciferin-luciferase reaction was comparable to that of the mollusc, *Pholas dactylus*, described by Dubois (1887), and that luciferin could be oxidized by oxidants or would oxidize spontaneously without light emission in absence of luciferase; that it was dialyzable and insoluble in ether and hydrocarbons, but soluble in lower alcohols and some other non-aqueous solvents. It was found that after oxidation, luciferin could be recovered, at least partially, by various procedures which add hydrogen to a molecule. Consequently the oxidation of luciferin was compared to the oxidation of a leuco-dye, such as methylene white to the dye, methylene blue. It was also established (Harvey, 1925–26) that luciferin undergoes photochemical destruction without light emission, an effect subsequently studied in detail by Chase and Giese (1940).

In the 1920's, additional properties of the *Cypridina* luminescent reaction were studied by Harvey and by Kanda, but the solutions were impure and nothing would be gained by reviewing the tests applied, or the procedures used in fractionation. One difficulty in testing for chemiluminescent substances is the extreme sensitivity of the luminescence test as compared with ordinary chemical reactions for organic substances. Luciferin may give a bright light with luciferase in concentrations where chemical tests are negative. It is possible to see the light from 1 part of dried powdered *Cypridinae* added to 400,000,000 parts of water, whereas a chemical test is considered sensitive if one part in a million can be detected.

A great advance in *Cypridina* luciferin chemistry was made by Anderson (1933), who introduced a quantitative method of measuring luciferin by the total light produced under standard conditions, and by the development of a method of purification (Anderson, 1935), which has been used ever since, and which has been the starting point for the more recent work to be described in this paper. The complicated procedure, mostly carried out in a hydrogen atmosphere

or at low temperature, involves extraction of dried *Cypridinae* with benzene to remove lipids, extraction with methanol to dissolve the luciferin, replacement of methanol with *n*-butanol, treatment with benzoyl chloride, partition between ether and water, hydrolysis of the benzoylated luciferin with aqueous HCl, partition of the freed luciferin with *n*-butanol, and storage of the butanol solution under hydrogen. At least two cycles of benzoylation must be carried out for greatest purity. In this paper, material so treated will be referred to as doubly cycled luciferin.

Removal of the butanol *in vacuo* leaves a resin-like amorphous brownish-yellow film with high luminescent activity. Each milliliter of the butanol luciferin solution contains from 0.05 to 0.115 mg. of solid and the purification of the material as measured in light units per unit of weight is about 2000 times that of the dried powdered *Cypridinae*. The purified luciferin is much more stable against spontaneous oxidation than are crude aqueous solutions, especially in dilute acid.

Anderson (1936) also demonstrated that only the product of spontaneous oxidation of luciferin, or the oxidation carried out with oxidants like ferricyanide, was reversible with reducing agents, and that the yield of luciferin on reduction of oxidized luciferin is only high (70%) if the reduction is carried out immediately after oxidation. If allowed to stand, little reduction occurs.

When luciferin is oxidized with luciferase, and reduction is immediately attempted, a slight amount of luciferin can be obtained, attributed by Anderson to reduction of a small amount of spontaneously oxidized luciferin. This type of oxidation proceeds simultaneously with the light-emitting oxidation in presence of luciferase and complicates the interpretation of reaction kinetics (Chase and Lorenz, 1945). Anderson (1937) also studied the relation between salt content and pH of the medium and total light emitted. Salts are not necessary for luminescence, although NaCl in 0.01 *M* concentration increases the total light 2.3 times, while KI decreases total light to 0.08 of that in distilled water. Kinetics of the *Cypridina* luminescent reaction, involving quantitative studies of the effects of temperature, light, pH, and various inhibitors have been carried out by Chase and collaborators during the years 1940–1952. A résumé of this work and

references to the literature will be found in the book of Harvey (1952). Recently the effect of hydrostatic pressure has been studied quantitatively by Bronk, Harvey, and Johnson (1952); the relation of oxygen concentration to light intensity by Hastings (1952).

Since Anderson's studies, a number of workers have published certain statements concerning the chemistry of luciferin, not all of which have been confirmed by later investigations. The earlier experiments have been reviewed by Harvey (1940) and later findings by Chase (1948), whose work has been especially concerned with the absorption spectrum, to be considered in a later section. It is not yet possible to specify the exact chemical structure of *Cypridina* luciferin. Early statements have little meaning since they were based not only on very impure material but also on reasoning from one luminous animal to another. It now seems certain that the luciferin of *Cypridina* is quite different from that of the firefly and from that of luminous bacteria; a considerable number of luminescent systems have probably arisen independently in evolution (Harvey, 1953).

Historically it is interesting to note that at various times Dubois regarded *Pholas* luciferin as a proteose, a nucleoprotein, or an albumin with acid properties. *Cypridina* luciferin has been called a peptone (Harvey, 1919), a phospholipid (Kanda, 1930), a polyhydroxybenzene derivative (Anderson, 1936; Korr, 1936), a hydroquinone-like compound with a ketohydroxy side chain (Chakravorty and Ballentine, 1941), a flavoprotein and pyridine nucleotide (Johnson and Eyring, 1944), and a chromopolypeptide (Mason, 1952b).

The hydroquinone type of structure was championed by the Dutch group of investigators (Kluyver, van der Kerk, and van der Burg, 1942; van der Kerk, 1942), particularly for bacterial luciferin, in the form of 1,4-dihydroxynaphthyl-2-hydroxymethylketone. It seems quite certain that this compound cannot be *Cypridina* luciferin, since no light appears when it is mixed with luciferase (Johnson, Rexford, and Harvey, 1949; Spruit, 1949), although related naphthohydroquinones have not been ruled out.

On the other hand, a number of investigators have suggested that flavins are important in bacterial luminescence. It has recently been found that luminescence results when reduced riboflavin or reduced riboflavin phosphate is added to cell-free extracts of luminous bac-



teria and a simple hydrogenated flavin has been designated bacterial luciferin (Strehler, Harvey, Chang, and Cormier, 1954). McElroy (private communication) has suggested that firefly luciferin is related to the pteridins.

Space does not permit a review of the complete history or the evidence for and against the above hypotheses. The important findings concerning the chemistry of *Cypridina* luciferin, facts which appear to have been established without question, may be summarized as follows: Analyses of the purest available luciferin, prepared by Anderson's procedure with two cycles of benzoylation, indicate the presence of nitrogen (Chase and Gregg, 1949), confirmed by the carbylamine test (Mason, 1952a) and by a ninhydrin positive reaction after (but not before) acid hydrolysis of material purified both by paper chromatography (Mason, 1952b) and by paper electrophoresis, as described in a later section. Recent studies, now reported for the first time, indicate no phosphorus in doubly cycled luciferin by the molybdenum blue reaction of Fiske and SubbaRow as modified by Sumner (1944). The reaction is capable of detecting one part in 5 million of phosphorus. No carbohydrate has been found by the anthrone test, capable of detecting one part in 900,000 of starch. Control tests with minimal amounts of phosphate and glucose indicate that the two reactions were carried out properly. Luciferin thus appears to contain C, H, O, and N as the primary constituents.

Anderson's benzoylation method of preparation, apparently yielding a compound nonluminescent with luciferase and nonoxidizable with oxygen, but hydrolyzed to active luciferin by HCl, indicates an OH, NH, or NH<sub>2</sub> group. Acetic anhydride will form a similar non-active acetyl-luciferin (Anderson, 1935).

The doubly cycled luciferin has been found to combine irreversibly with cyanide, possibly indicating cyanhydrin formation with aldehyde or ketone groups (Giese and Chase, 1940). With azide, luciferin forms a reversible combination, possibly analogous to the reaction of hydrazoic acid with hydroquinones forming azidohydroquinones and finally aminoquinones, after liberation of nitrogen (Chase, 1942). Chase (unpublished) found no reaction with 2,4-dinitrophenylhydrazine.

Additional chemical tests have been made by Mason and Davis (1952), which led them to the conclusion that a ketophenol and a

quinonoid structure are ruled out, but the amino group appears to be present, and it is possible that the oxidation-reduction change in luciferin involves the NH group.

Anderson (1936) and Korr (1936) by an indirect method have placed the redox potential of luciferin at about +0.26 at pH 7. The rapid oxidation by ferricyanide has been made use of by Chase (1949) in estimating that the combining weight of luciferin is between 250 and 570, while a similar calculation from combination with cyanide indicated 800 to 2400 (Giese and Chase, 1940). Such calculations are subject to revision, depending on the amount of impurity present.

Recent methods of purification and approach to the structure of *Cypridina* luciferin such as infrared spectroscopy, chromatography, and acid hydrolysis have been initiated by Mason (1952b) and Mason and Davis (1952). Together with a fourth approach, paper electrophoresis, they will be discussed subsequently. Infrared spectroscopy, carried out with thin films of dried doubly cycled luciferin, has revealed strong absorption bands at 3250, 2825, 1680, 1625, and 1510  $\text{cm}^{-1}$ , collectively indicating the amide bond as it occurs in peptides or in cyclic ureides. Acid hydrolysis of paper chromatographed luciferin has yielded a yellow pigment and some eight amino acids, a result which led Mason (1952b) to the designation of *Cypridina* luciferin as a chromopolypeptide of a cyclic nature. A polypeptide structure of luciferin would place it in the group with a number of naturally occurring biological compounds, particularly the antibiotics gramicidin and polymixin, but with the addition of a color group. The solubilities of *Cypridina* luciferin are surprisingly similar to the cyclopeptide gramicidin, with the one exception that luciferin is more soluble in water.

The primary objective of the current work has been to isolate pure *Cypridina* luciferin and establish a criterion of purity, while the ultimate goal is the isolation of luciferin in sufficient quantity to permit the elucidation of the structure by physical and chemical means. Three main lines of investigation have been pursued in an attempt to isolate pure luciferin: (1) paper chromatography, (2) separation on ion-exchange resins and other adsorbents, and (3) filter paper electrophoresis. Spectral absorption measurements and fluorescence observations have been made on the highly purified luciferin, as well

as quantitative determinations of the amino acids present after hydrolysis by acid. Methods and results are described below.

### Chromatographic Purification

The starting point for chromatographic procedures was *Cypridina* luciferin prepared by two cycles of benzoylation according to the method of Anderson (1935). The final butanol solution of luciferin was stored under an atmosphere of hydrogen and aliquots were withdrawn as required. The butanol could then be removed by evacuation and the residue redissolved in methyl alcohol for work with paper chromatography and in the appropriate buffers for work with paper electrophoresis and ion-exchange resins. All these methods, as well as measurement of the absorption spectrum of doubly cycled luciferin, have revealed the presence of at least several impurities.

Mason and Davis (1952) were able to chromatograph *Cypridina* luciferin on No. 1 Whatman paper, using the ascending technique. They worked at room temperature in a hydrogen atmosphere saturated with the vapor phase of the developing solvent. They reported that doubly cycled luciferin was separable by several solvents into what they designated as alpha and beta luciferins possessing the following  $R_f$  values, respectively: chloroform, 0 and 0.25; *n*-butanol, 0 and 0.55, and *n*-butanol saturated with water; 0.8 and 0.8. Both luciferins were yellow but did not fluoresce. Their chromatographic studies did not reveal any impurities, either by ordinary or ultraviolet light, or by any of various tests tried.

The preceding evidence may not necessarily indicate the existence of two kinds of luciferin, for in most of the present work only one luciferin has been observed, as manifested by a single, intense luminous area on the paper when treated with luciferase. Occasionally after paper electrophoresis and more often with paper chromatography, a less intense luminous spot was detected at the origin, but this might be ascribed either to luciferin strongly adsorbed by the paper or to undissolved particles of luciferin, or both. At other times extremely weak luminescence has been observed at the solvent front after paper chromatography, but this is probably due to luciferin bound to impurities moving at the front. Luminescence at the origin was nearly always observed in paper chromatography where separa-

tions were poor as evidenced by either high or low luciferin  $R_f$  values accompanied by considerable tailing. At no time, however, have two luminous regions of the same area and intensity been observed; when two regions *have* been observed, one has always been at the origin.

The work of Mason and Davis was repeated in every detail. For chloroform, the principal luminescent area was at  $R_f = 0$ , with a broad, unbroken band extending forward to about  $R_f = 0.14$ . When *n*-butanol was used, the principal luminescent region was at  $R_f = 0.77$  and a very small, less intense spot occurred at  $R_f = 0$ . The diameter of the spot at  $R_f = 0$  was practically the same as that of the luciferin spot at the origin before chromatography. With *n*-butanol saturated with water, the  $R_f$  of luciferin was about 0.88; there was no other luminous region. In addition to these solvents two other solvents, phenol and collidine (2,4,6-trimethylpyridine), each saturated with water, were also tried with little success. With the former, the residual phenol remaining in the paper after drying strongly inhibited the luminescent reaction with luciferase, whereas with the latter, rapid destruction of luciferin occurred.

With Whatman No. 3 filter paper, the ascending technique was again employed and the chromatography run in the cold room at 2.0–2.5° C for a period of four or more hours. Doubly cycled luciferin in methanol was used to spot the paper. Solvents tried in various combinations were benzene, *n*-propanol, water, acetone, *n*-butanol, ethylene glycol, *n*-amyl alcohol, *n*-hexyl alcohol, and ethyl ether. They gave from very poor to fair separations and considerable streaking occurred in most of them. The best chromatography was obtained with a developing solvent consisting of ethyl acetate, ethyl alcohol, and water (5,2,3 by volume, upper layer used). The paper, spotted with doubly cycled luciferin, stood in the developing chamber, in contact with the vapor, for one-half hour before lowering into the developing solvent. After the solvent front had moved a distance of about 26 cm (4 hours), the paper was removed from the chamber and dried in air, this process taking only a few minutes. On visual examination, the paper showed two main areas: a yellow-brown one at the solvent front and a yellow one between the front and the origin (see Fig. 1). When examined under a Mineralite lamp, two brightly fluorescent areas were observed: one of yellow fluorescence,

identical in position to the yellow color ( $R_f = 0.5-0.6$ ), and one of blue fluorescence ( $R_f = 0.2$ ). The yellow-brown area at the solvent front showed only a weak, diffuse yellow fluorescence. On moistening the paper with luciferase solution, a single intense luminous area was observed which corresponded exactly to the area of the yellow color and yellow fluorescence. The yellow color, yellow fluorescence, the luminescence with luciferase and the  $R_f$  of 0.5-0.6 in this developing solvent can, therefore, be considered characteristic properties of *Cypridina* luciferin. Of these, luminescence with the enzyme is the ultimate criterion. The other materials on the paper are presumably im-

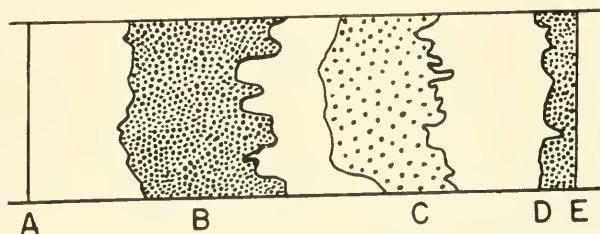


FIG. 1. Diagram showing paper chromatogram of doubly cycled luciferin developed with ethyl acetate-ethyl alcohol-water mixture. A represents origin; band B, an impurity, shows only blue fluorescence; band C, luciferin, shows yellow color and yellow fluorescence; D, a yellow-brown impurity; and E represents solvent front.

purities. In similarly developed chromatograms, the yellow luciferin area of the paper was cut out and eluted with methyl alcohol. The yellow methanol solution was evacuated to dryness and the residue used for spectrophotometric studies (to be discussed in a later section) and for amino acid analysis by the chromatographic method of Moore and Stein (1951).

Efforts have recently been made to isolate luciferin by ion-exchange resins in amounts larger than heretofore obtainable with paper chromatography and paper electrophoresis. Fine mesh Dowex 50 of various cross-linkages was tried in a column 2.26 sq cm  $\times$  9.0 cm. Luciferin was taken up readily from 0.1 N HCl at room temperature (23° C) in a system in which air had been replaced by pure hydrogen. Elution was attempted, using successively oxygen-free 0.1 N HCl, 1 N HCl, 3 N HCl, saturated NaCl, phosphate buffer pH 6.8,



0.2 M disodium hydrogen phosphate (pH 8), and 0.1 N NaOH. Elution occurred only with alkaline solutions of pH 8 or greater, but here luciferin lost its activity so rapidly that alkaline eluants appeared undesirable.

A carboxylic acid resin, XE-97 (Rohm and Haas), an analog of IRC-50, was also tried in fine mesh form in a column 2.26 sq cm  $\times$  17.5 cm at 2.0–2.5° C. Luciferin was readily taken up from pH 6.4 phosphate buffer, forming a thin yellow fluorescent band at the top of the column. One-cubic centimeter fractions were collected with a fraction collector. The following eluants were tried without success: pH 6.4 phosphate buffer, pH 5.0 citrate buffer, and pH 3.4 citrate buffer. None of the fractions, adjusted to pH 6.8, gave light when tested with luciferase, and the yellow fluorescent band remained at the top of the column. When pH 3.4 citrate buffer containing 25% by volume of ethyl alcohol was used, the yellow fluorescent band moved slightly, but the effluent fractions gave no light when tested with luciferase (after making the pH 6.8 and diluting to minimize luciferase inhibition by the alcohol).

The yellow band of the resin column was carefully removed and transferred to a 15-ml centrifuge tube where, after centrifuging off the buffer, the resin was washed with methyl alcohol and then centrifuged. A clear yellow supernatant was obtained which gave brilliant luminescence with luciferase. Some of this was evacuated to dryness and the yellow residue redissolved in 0.1 N HCl. The absorption spectrum of this solution resembled that of those obtained by paper chromatography and also those from paper electrophoresis, although it showed slightly greater density at the short wavelengths. Using spectral absorption as a criterion, it is obvious that chromatographic procedures have resulted in removal of many extraneous substances and the preparation of luciferin in a high degree of purity.

Further attempts have been made to isolate luciferin in larger amounts by employing column-partition chromatography techniques. Preliminary experiments with powdered cellulose columns operated in the cold room at 2.5° C have yielded rather good separation of luciferin from crude extracts of the material. Water represented the stationary phase, and a mixture of ethyl acetate and ethyl alcohol, saturated with water, made up the mobile phase. The crude luciferin solution



was prepared by extracting fat-free *Cypridina* powder with methyl alcohol, then removing the alcohol by evacuation, extracting the luciferin residue with butyl alcohol, removing this alcohol by evacuation, and finally redissolving the luciferin in the mobile phase mixture for placement on the column. The developed band of luciferin could then be followed on the column by its yellow color and yellow fluorescence and could be collected at the appropriate time with a fraction collector. The luminescence with luciferase served to identify this luciferin. There is some evidence from spectral measurement that the luciferin isolated by the above procedure from the crude extract is different from the luciferin prepared by the Anderson's benzoylation method.

### Electrophoretic Purification

Filter paper electrophoresis of doubly cycled material was also used to isolate luciferin for spectrophotometric comparison with that obtained by paper chromatography. The electrophoretic method was essentially that of Kunkel and Tiselius (1951). A 0.1 M  $\text{NaH}_2\text{PO}_4$ - $\text{H}_3\text{PO}_4$  buffer of pH 2.55 and ionic strength 0.1 was used to dissolve the luciferin. A potential gradient of approximately 5.9 volts/cm and 6.4 ma was applied to a double layer of Whatman No. 3 paper for 14 hours at 10° C. The acid pH was selected because luciferin is more stable at low pH (Anderson, 1936). Dextran and Armour's crystalline bovine albumin were run concurrently and localized in a strip of the paper after electrophoresis with 1% bromophenol blue in absolute ethyl alcohol saturated with mercuric chloride. After dyeing the paper was washed with absolute methyl alcohol, leaving the albumin blue. Luciferin is presumably washed out by the methyl alcohol in which it is extremely soluble. Dextran indicated the degree of electro-osmotic flow of the buffer.

After electrophoresis, a definite area on another strip of the paper showed a yellow color, a yellow fluorescence in the ultraviolet light of a Mineralite lamp, and luminesced with luciferase. As in the case of chromatography with ethyl acetate, only one intense luminescent area was observed. Luciferin, like albumin, was positively charged at this pH. The ratio of the distance moved by luciferin to that moved by albumin was approximately 0.34 (3.4 cm for luciferin, 10 cm for

albumin). The luciferin area was eluted with methyl alcohol, the yellow solution evacuated to dryness, and the residue dissolved in 0.1 M HCl for measurements of the absorption spectrum (to be discussed in the next section).

Paper electrophoresis of luciferin was also carried out at room temperature and pH 4.0 (acetic acid/sodium acetate buffer), pH 5.8 (acetic acid/pyridine buffer), and pH 8.9 (veronal/veronal sodium buffer). At these pH values, two intense blue fluorescent spots, probably the same blue fluorescent substance seen in paper chromatography, were observed moving toward the negative pole. At pH 4.0 and pH 5.8, the luciferin was still positively charged, although bovine albumin was positively charged at the lower pH but negatively charged at the higher pH. At pH 8.9, the inhibition of luciferase by sodium barbiturate and the auto-oxidation of luciferin (resulting in low light production) were too great to localize the position of the migrating luciferin.

### Absorption Spectrum

The first indication that luciferin possessed specific spectral absorption which might prove useful in its isolation and identification was the observation (Chase, 1940) that neutral aqueous solutions had an absorption band at about 435  $m\mu$  which was replaced during exposure to air by another at about 465  $m\mu$ . The latter subsequently disappeared, leaving the solution colorless to the eye. A yellow color had, indeed, been associated with *Cypridina* luciferin since it was first studied by Harvey (1917), and Anderson (1935) had never been able to dissociate color from the ability to give light with luciferase, even after as many as three cycles of purification by his method.

The visible absorption band of an aqueous luciferin solution\* and the changes which it underwent during exposure to air made it evident that information on luciferin structure might come from ultraviolet absorption spectrophotometry, and that the spectrum would certainly be useful as a criterion of purity. Chase and Brigham (1951) obtained the first reliable measurements of the ultraviolet absorption

\* The luciferin used in the work to be described in this section was in all cases obtained by two cycles of Anderson's (1935) purification procedure before being subjected to further purification methods.

spectrum of doubly cycled *Cypridina* luciferin, which had been washed with benzene to minimize ultraviolet-absorbing impurities. Luciferin itself is insoluble in benzene. The spectra were measured in pH 6.8 phosphate buffer solution, using the Beckman spectrophotometer, and are shown in Fig. 2. The heavy line represents the solution immediately after dissolving the luciferin. As shown earlier (Chase, 1940), an initial absorption maximum in the visible region at about

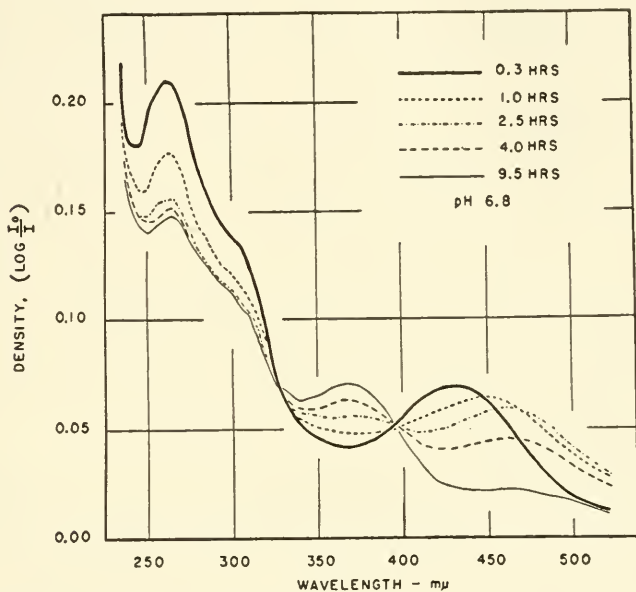


FIG. 2. Absorption spectra of a solution of doubly cycled and benzene-washed luciferin, measured in pH 6.8 phosphate buffer. The heavy line spectrum was obtained as soon as possible after the solution had been prepared. The solution, standing exposed to air, at room temperature, was then remeasured at the times indicated. Characteristic of a luciferin solution under these conditions is the shift of the visible absorption band from a maximum at 435 to one at 465  $m\mu$ . Solutions of pH 1.0 do not show this shift (see Fig. 3).

435  $m\mu$  is rapidly replaced by one at about 465  $m\mu$  and the latter then almost entirely disappears during exposure of the solution to air. In the ultraviolet the initial spectrum has a pronounced absorption peak at 265  $m\mu$ , and a shoulder at about 310  $m\mu$ . On exposure to air the absorptions at 265 and 310  $m\mu$  decrease and, simultaneously, a new band appears at about 365  $m\mu$ . Isosbestic points exist at 330 and

395  $m\mu$ . The absorption peak at 265  $m\mu$  could be responsible for photochemical changes which have been observed in luciferin after exposure to ultraviolet light (Chase and Giese, 1940).

The stability as well as the form of the absorption spectrum are very dependent upon the hydrogen ion concentration. The spectrum is relatively stable at pH 1.0 but becomes rapidly less so at higher pH's. For this reason many measurements of the visible and ultraviolet spectra have been made under various experimental conditions in an effort to establish a curve which would with certainty represent luciferin. None of these measurements has previously been published. Recently, application of the techniques of paper chromatography and paper electrophoresis has produced luciferin that is very much purer than any available previously. The details of the methods have already been described. By means of micro attachments for the Beckman spectrophotometer (Lowry and Bessey, 1946), we have now measured and compared absorption spectra of luciferin isolated by these methods. Since these purification procedures yield products having one and the same absorption spectrum which, furthermore, undergoes identical changes on exposure to air and treatment with base or acid, it would seem justified to assume a high degree of purity for the luciferin.

In Fig. 3, five sets of absorption spectra are shown. Two different, doubly cycled luciferin preparations are represented, made independently by two of us, using different batches of *Cypridinae* and slightly different procedures. These preparations will be designated as I and II. The absorption spectra shown in Figs. 3A, 3B, and 3C are from preparation I; those in Figs. 3D and 3E, from preparation II. In all cases the solid line represents the spectrum measured as soon as possible after dissolving the material in 0.1 N hydrochloric acid, with minimal exposure to air. The dash line and dotted line show the spectra of the same solutions after exposure to air for the times indicated.

Figure 3B gives spectra of a luciferin solution which was subjected to no further purification than two cycles of Anderson's (1935) procedure. Considerable general ultraviolet absorption is evident, but inflections in the curves and definite changes during exposure to air are quite apparent. The spectra in Fig. 3C are from a solution of luciferin isolated from preparation I by paper chromatography, while

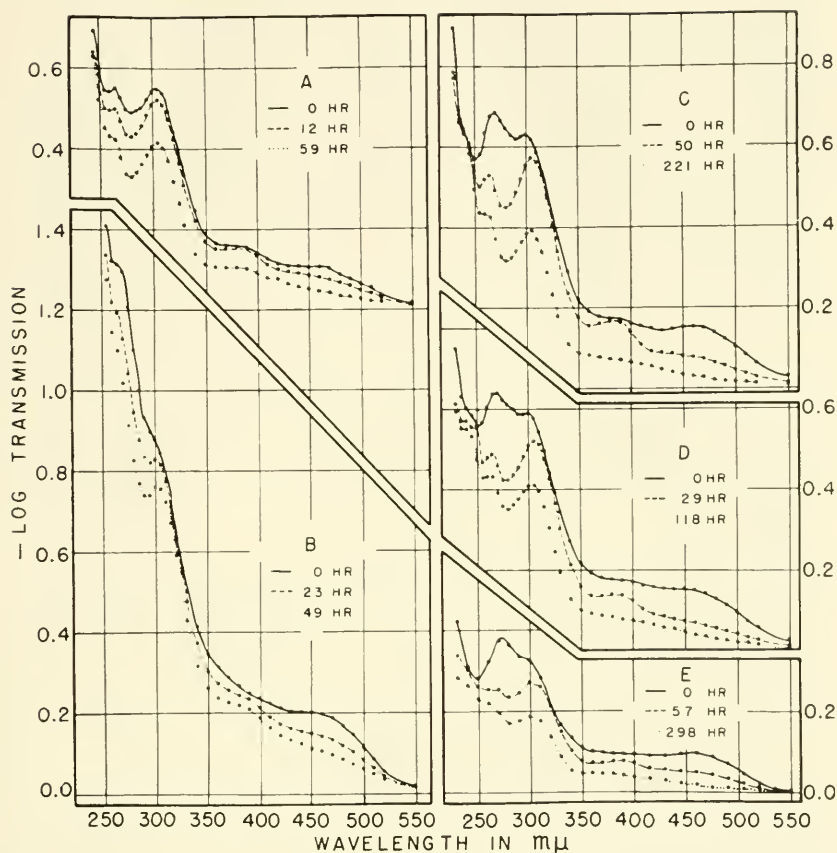


FIG. 3. Absorption spectra of 0.1 N HCl solutions of doubly cycled luciferin, purified further in various ways as described in the text. In each set of spectra the solid line represents the solution measured as soon as possible after being prepared, with minimal exposure to air, whereas the other two curves were measured after the solution had been exposed to air for the times indicated. A, B, and C are from one luciferin preparation, and D and E from another. It is apparent from inspection of these five sets of absorption spectra (particularly C, D and E) that they are similar in form and in the changes which they undergo on exposure of the luciferin solutions to air. They may be presumed to represent luciferin. See the text for further details.

those of Fig. 3A represent a solution of luciferin isolated from the same preparation by paper electrophoresis.

Figure 3E shows the spectra of preparation II, washed with benzene after the last step in Anderson's procedure, but not otherwise treated.



In Fig. 3D are drawn the spectra of this same preparation after further purification by paper electrophoresis.

It is at once apparent on inspection of the five sets of absorption spectra in Fig. 3 that they all show, not only the same absorption maxima, but also the same changes when the solutions stand in contact with air at room temperature. Even in the presence of considerable impurity, as in Fig. 3B, the same absorption maxima (represented by inflections) and the same changes in the spectrum are easily discernible. The definite difference between the initial spectrum in Fig. 3A and those of Figs. 3C, 3D, and 3E is undoubtedly due to the fact that, in the first case, a longer time elapsed between eluting the material from the paper and measuring the initial spectrum. It is practically certain that the changes observed in the absorption spectra of luciferin solutions during exposure to air are the result of oxidation of some sort.

Disregarding Fig. 3B, the initial spectra in Figs. 3A, 3C, 3D, and 3E are practically identical. They have an absorption maximum at  $265\text{ m}\mu$ , a smaller peak (or shoulder) at about  $310\text{ m}\mu$  and a broad absorption band in the visible region, centering at about  $465\text{ m}\mu$ . On standing, exposed to air at room temperature, the  $265\text{-m}\mu$  peak becomes less, the peak at  $310\text{ m}\mu$  becomes much more pronounced (is possibly unmasked), a new band appears at  $380\text{ m}\mu$  and much of the visible absorption disappears. Finally, as the dotted line curves show, the  $380\text{-m}\mu$  band and practically all visible absorption disappear and, at the same time, there is a further decrease in the absorbance of the bands at  $265$  and  $310\text{ m}\mu$ . By this time the spectrum has become practically stable.

It seems reasonably certain that *Cypridina* luciferin has now been isolated in a rather high state of purity and that, consequently, the absorption spectra of Fig. 3 may be characteristic of luciferin at pH 1.0 and of the changes which it undergoes at this pH on exposure to air.

A comparison of Figs. 2 and 3 shows that the spectrum of a luciferin solution is not only less stable at pH 6.8 than at pH 1.0, but also that the changes on exposure to air are different. Clearly, the hydrogen ion concentration affects one or more components of this system. Furthermore, dilute hydrochloric acid solutions of doubly cycled lucif-



erin are definitely yellowish to the eye but become colorless in excess of sodium hydroxide. The disappearance of the visible absorption band at  $465\text{ m}\mu$  is responsible for this color change and even more striking changes occur in the ultraviolet region of the spectrum. If the solution is immediately made acid again, the yellow color partly returns. There are evidently acid-base changes in absorption, complicated by rapid oxidation changes especially marked in alkaline solution, and these will now be described.

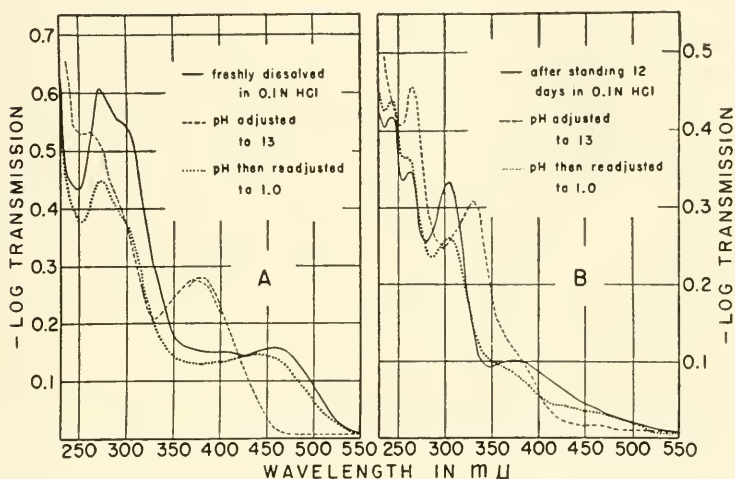


FIG. 4. In A, the dash line shows the absorption spectrum of a luciferin solution adjusted to pH 13 soon after having been prepared in 0.1 N hydrochloric acid and before significant oxidation has occurred in the acid solution. An unstable absorption peak appears at  $380\text{ m}\mu$ , which shifts rapidly toward shorter wavelengths. The dotted line curve shows the spectrum of the same solution after having been readjusted to pH 1.0. In B, the dash line represents the spectrum of a luciferin solution adjusted to pH 13 after having stood exposed to air at pH 1.0 for twelve days, until presumably the luciferin was completely oxidized. The alkaline spectrum, which is relatively stable, shows a new absorption peak at  $330\text{ m}\mu$ . See the text for details and discussion.

As shown in Fig. 3, a freshly prepared 0.1 N hydrochloric acid solution of luciferin, isolated by paper chromatography, has a pronounced absorption peak at  $265\text{ m}\mu$  and a shoulder at about  $310\text{ m}\mu$ . There is no specific absorption in the long-wavelength region of the ultraviolet. On adjusting the pH of such a solution from 1.0 to 13 with sodium

hydroxide, a striking change occurs in the spectrum. Much of the far ultraviolet absorption and practically all in the visible disappear and a new peak is seen, maximal initially at about  $380\text{ m}\mu$ . This new peak apparently shifts when the solution is exposed to air at room temperature and in about thirty minutes becomes stable, with a maximum at about  $355\text{ m}\mu$ . The initial shift is quite rapid.

These changes are shown in Figs. 4A and 5. In Fig. 4A, dash line curve, the first measurement was made at  $550\text{ m}\mu$ , and the measure-

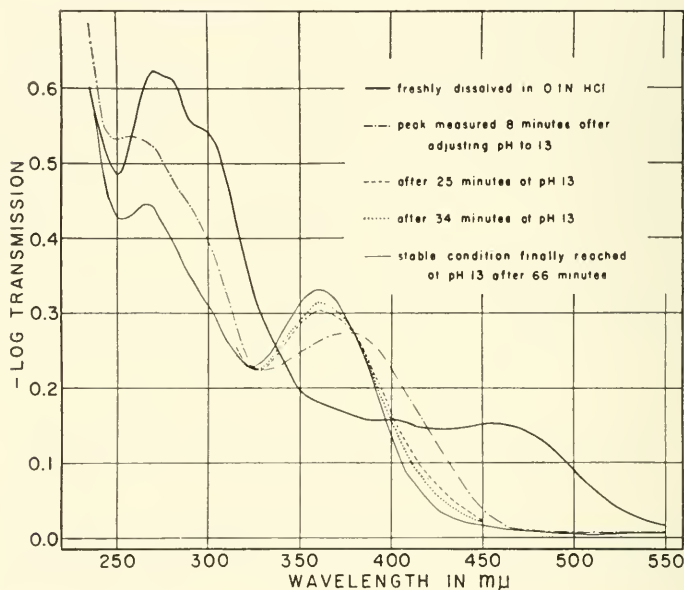


FIG. 5. An experiment designed specifically to show the rather rapid change in the absorption spectrum of a freshly prepared 0.1 *N* hydrochloric acid solution of luciferin on adjusting the pH to 13 with sodium hydroxide. The most prominent feature of the new alkaline spectrum is appearance of an absorption peak at  $380\text{ m}\mu$ , which rapidly shifts until it becomes stable at about  $355\text{ m}\mu$ . The form of the stable alkaline spectrum is shown by the light solid line curve.

ments were then continued toward shorter wavelengths, to  $365\text{ m}\mu$ . The next was then made at  $400\text{ m}\mu$ , after which they were again continued in the direction of the shorter wavelengths. The rapid shift of the  $380\text{-m}\mu$  absorption peak is quite evident from this sequence of measurements.

On adjusting the pH of such an alkaline luciferin solution back to pH 1.0 from 13, the absorption band at  $355\text{ m}\mu$  largely disappears and some of the specific absorption of the acid solution in the visible region and at  $265$  and  $310\text{ m}\mu$  is restored. A spectrum measured under such conditions is shown in Fig. 4A (dotted line curve), and it is apparent that the final acid absorption spectrum resembles qualitatively that of the acid luciferin solution as originally prepared, although the densities are considerably decreased. An obvious interpretation of these results (although not necessarily the only one) might be that during the interval when the luciferin was exposed to dissolved oxygen at pH 13 an appreciable amount was oxidized, so that upon making the solution acid once more, and thereby restoring the original form of the absorption spectrum, much of the absorbance of the peaks had been lost. The slight qualitative differences between the initial and the final spectra might be attributed to contribution by the spectrum of the oxidized form of the material.

It is evident from the following experiment that reaction with dissolved oxygen certainly plays a role in the above changes. If all oxygen is displaced from an acid luciferin solution immediately after its preparation, by passing pure hydrogen through the solution in the absorption cell, and if such an oxygen-free solution is then adjusted to a pH of 13, an absorption peak centering at  $380\text{ m}\mu$  is found and this peak is perfectly stable as long as the solution remains oxygen free.

As has already been demonstrated in some detail, the absorption spectrum of aqueous luciferin solutions undergoes specific changes on standing exposed to air, and these changes are probably due to oxidation of the luciferin. If a luciferin solution of pH 1.0 is allowed to stand, exposed to air, until the spectrum has reached a practically stable condition, and if the pH is then adjusted to 13 with sodium hydroxide, a different absorption spectrum is obtained than when freshly prepared luciferin solutions of pH 1.0 are adjusted to pH 13. It will be recalled that fresh solutions show a new band at  $380\text{ m}\mu$  which rapidly shifts to a stable position at about  $355\text{ m}\mu$ . On the other hand, the solution which has stood at pH 1.0 until its spectrum is stable, and is then adjusted to pH 13, shows a band which is relatively stable at  $330\text{ m}\mu$  as shown in Fig. 4B. This result indicates that a

change in the structure of the molecule may have occurred during its slow oxidation at pH 1.0. If the solution now be made acid again, some of the original acid form of the spectrum is restored (dotted line curve of Fig. 4B).

It is quite clear that solutions of luciferin purified in several different ways possess very specific absorption in the visible and ultraviolet regions of the spectrum. Furthermore, the spectrum undergoes pronounced changes under various experimental conditions. Were this absorption spectrum less complicated, one might feel optimistic about the identification of *Cypridina* luciferin from its spectrum alone. Actually, the interpretation of an absorption spectrum in terms of chemical structure can be very difficult and frequently impossible unless other physical and chemical data are available. To date we have not been able to find in the literature any known compound whose absorption spectrum possesses the properties described here, and at the present time it is not possible on the basis of these measurements to make definite statements regarding the type of organic molecule to which *Cypridina* luciferin belongs. The spectrum may well prove of value in the ultimate identification, in conjunction with other information which may become available. Certainly, the ultraviolet absorption spectrum is proving an excellent criterion of purity and, in this way at least, is aiding greatly in the isolation of this extremely unstable compound.

### Fluorescence

It has usually been stated that *Cypridina* luciferin is nonfluorescent. Certainly there is no marked fluorescence in solution characteristic of such substances as quinine sulfate, oxidized flavins, acid luminol solution, and various dyes. In this respect *Cypridina* luciferin differs from that of fireflies, which is highly fluorescent, bluish violet in weak acid and yellow-green in alkali (Strehler and McElroy, 1949).

However, it had been observed (Harvey and Chase, unpublished, 1941) that the brownish, solid, doubly cycled luciferin fluoresced weakly yellow in the light from a mercury arc filtered through a Wood nickel oxide filter. This light consists principally of radiation in the near ultraviolet, the 365-m $\mu$  line. There was, however, no certainty that the fluorescence came from luciferin.

It has been pointed out in previous sections that paper chromatography and paper electrophoresis techniques demonstrate that in the doubly cycled luciferin there are at least two fluorescent substances, one of which is luciferin, yellow fluorescent, and the other an impurity, blue fluorescent. For further study of luciferin fluorescence, the yellow fluorescent region of the filter paper was eluted with methanol, the solvent removed *in vacuo*, and the residue dissolved in 0.1 N HCl. In a beam of white light, observed from the side, no fluorescence can be detected, indicating a relatively weak intensity, but in the Wood light (maximum energy 365  $m\mu$ ) and also in a Mineralite (ultraviolet without the visible; maximum energy 253.6  $m\mu$ ) the luciferin solution is yellow fluorescent. In the light from a "Purple ultra" incandescent bulb the fluorescent color was green to bluish green, probably due to a combination of the yellow fluorescence and the blue light transmitted by this bulb. Dilution of the 0.1 N HCl luciferin with 9 parts of water nearly abolishes the fluorescence, indicating its low intensity. Paper chromatographed luciferin is also yellow fluorescent when dissolved in methanol and butanol. It is clear that *Cypridina* luciferin does exhibit fluorescence,\* although it is far less bright than the fluorescence of many other chemiluminescent substances.

The effect of acid-base change and oxidation of chromatographed luciferin in aqueous solution was studied by preparing three tubes with luciferin in 0.1 N HCl: leaving one tube acid, neutralizing another with NaOH, and making a third alkaline with NaOH to about pH 13. Table I shows the relative fluorescence of the fresh, active luciferin and of the material allowed to stand for 14 days until the luciferin had completely oxidized.

It is apparent from the observations noted in the table that the fluorescence of a 0.1 N HCl solution of luciferin is very much less after it has been exposed to air until completely oxidized than when freshly made up. This can quite probably be interpreted in terms of the decrease of the ultraviolet absorption of such a solution which

\* In certain solvents (e.g., ethanol) at room temperature, *Cypridina* luciferin exhibits an apparent "phosphorescence" after irradiation with ultraviolet light. This effect is believed to be a sustained chemiluminescence induced by the radiation (see Tsuji and Harvey, 1954).



was demonstrated in the last section (see Fig. 3, for example). The fact that, even in the freshly prepared acid luciferin solution the fluorescence is only slight, compared with that of such compounds as riboflavin, may be in part due to absence of pronounced absorption bands in the near ultraviolet region of the spectrum (i.e., 330–400  $m\mu$ ). On the other hand, quenching phenomena may be involved, as in the case of flavins combined with protein (see Weber, 1950).

TABLE I

Fluorescence of Chromatographed Luciferin and Completely Oxidized Luciferin

Preparation	Color	Fluorescence in	
		Mineralite	Wood's ultraviolet
<i>Luciferin</i>			
On paper	Yellow	Bright yellow	Yellow
0.1 <i>N</i> HCl	Yellow	Yellow	Yellow
Neutral	Yellow	Yellow	Yellow
0.1 <i>N</i> NaOH	Colorless <sup>a</sup>	Deep yellow	Yellow <sup>b</sup>
<i>Oxidized Luciferin</i>			
On paper	Colorless	Pale yellow	Pale yellow
0.1 <i>N</i> HCl <sup>c</sup>	Colorless	Pale yellow	Pale yellow
Neutral <sup>c</sup>	Colorless	Pale yellow	Pale yellow
0.1 <i>N</i> NaOH <sup>c</sup>	Colorless	Very pale yellow	Very pale yellow

<sup>a</sup> The color returns to a less intense yellow on immediate neutralization.

<sup>b</sup> On first making alkaline, the yellow fluorescence is relatively more intense than after standing exposed to air for 15 minutes.

<sup>c</sup> Studied in a nonfluorescent cellophane tube after 3 weeks when no light appeared on mixing with luciferase.

When a freshly prepared 0.1 N hydrochloric acid solution of paper chromatographed luciferin is made alkaline (pH 13) with sodium hydroxide and is then irradiated with the Wood light, there is at first a slight enhancement of the fluorescence compared with that given by the freshly prepared acid solution. The fluorescence rapidly decreases, however, as the alkaline solution stands, and becomes relatively faint. This effect can perhaps be explained by the changes which occur in the absorption spectrum of such a luciferin solution upon adjusting its pH to 13. It will be recalled that a prominent, new absorption band appears at 380  $m\mu$  when such a luciferin solution is



first made alkaline, but that on standing exposed to air, this band rapidly shifts to a new maximum at about  $355\text{ m}\mu$  passing through the region  $365\text{ m}\mu$  of great intensity in the Wood light (see Fig. 4A and Fig. 5). It is quite possible that the initial enhancement of the fluorescence when the solution is made alkaline is due to absorption in the  $365\text{-m}\mu$  region, and its subsequent diminished intensity is because of the appearance of the  $355\text{-m}\mu$  band at a shorter wavelength where less energy is emitted by the Wood light.

On the other hand, an oxidized acid luciferin solution that is then adjusted to pH 13 shows very faint fluorescence when irradiated with the Wood light. This seems quite understandable in view of the near ultraviolet absorption spectrum of such a solution, as described in the last section and shown in Fig. 4B. It will be recalled that such a solution exhibits a rather narrow absorption band centering at about 330 millimicrons. Very little energy would be available at this wavelength in the Wood light for fluorescence excitation. There does, therefore, appear to be consistency between the results of the experiments on fluorescence and those involving the absorption spectrum of luciferin solutions under various conditions. A quantitative determination of the spectral distribution of the fluorescence of solutions of paper chromatographed luciferin should be of value for the identification of this compound and such measurements are contemplated. Of course, it is always possible, as was stated earlier in this section, that if luciferin is a chromopolypeptide, as suggested by Mason (1952b), the spectral distribution of fluorescence might be so affected by the presence of the constituent amino acids as to make any interpretation in terms of chemical structure extremely difficult.

### Hydrolysis of Luciferin

Mason (1952b) has reported that his alpha and beta luciferins are chromopolypeptides, on the following grounds: (1) alpha luciferin was convertible to beta luciferin under certain experimental conditions; (2) the infrared spectrum of beta luciferin indicated amide bonds as they occur in peptides and cyclic ureides; (3) hydrolysis of beta luciferin yielded a number of amino acids, including an unidentified ninhydrin positive substance and a yellow pigment; (4) beta luciferin gave a positive N-chloroamide test (Rydon and Smith, 1952),

thus indicating the presence of either an amino acid, protein, cyclic peptide, diketopiperazine, acylated amino acid, or acylated peptide; and (5) beta luciferin gave a positive dye retention test (Robinson and Fehr, 1952) for proteins and peptides. The amino acids obtained from 24-hour hydrolysis of beta luciferin with 4 N HCl at 135° C were identified by two-dimensional paper chromatography and microbiological assay as follows: glycine, threonine, proline, lysine, aspartic acid, glutamic acid, and either leucine, isoleucine, or phenylalanine.

In view of Mason's findings, the following experiments were undertaken, using 28 mg of doubly cycled luciferin. The absorption spectrum of this material indicated the presence of some impurity, but the following tests were negative: biuret, ninhydrin, anthrone (for carbohydrates), and molybdenum blue (for phosphorus after complete digestion). Quantitative protein determination by the method of Lowry *et al.* (1951) indicated that this luciferin preparation was 28% protein or polypeptide, calculated in terms of equivalence of crystalline bovine albumin. This doubly cycled luciferin was hydrolyzed by refluxing with 6 N HCl (ninhydrin negative) for 16 hours according to the method of Stein and Moore (1948).<sup>\*</sup> The hydrolyzate after filtration had a clear golden-orange color, was moderately fluorescent (ultraviolet excitation) and gave weak luminescence with luciferase. It also showed a strong positive ninhydrin test according to the method of Moore and Stein (1948). The ninhydrin color value obtained indicated an equivalence of 35% leucine. The hydrolyzate was analyzed for amino acids, using both the long and short Dowex 50 columns, by the method of Moore and Stein (1951). Very good separations of the amino acids were obtained. Recovery of ninhydrin color value, expressed as equivalence of leucine introduced into both columns, was 107%. By this technique, the following substances were found in the luciferin hydrolyzate: taurine, aspartic acid, threonine, serine, sarcosine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, ammonia (from degradation of amino acids and amides), histidine, and arginine. The presence of beta alanine, tryptophane, ethanolamine, hydroxylysine, and ornithine was uncertain. On the other hand, there

<sup>\*</sup> It is a pleasure to acknowledge the collaboration of Dr. Harold H. Williams of Cornell University in the amino acid analysis.

was no cysteic acid, urea, hydroxyproline, citrulline, cystine (nor cysteine), alpha-amino-*n*-butyric acid, and glucosamine. The relative abundance of the amino acids is shown in Figs. 6 and 7.

In addition, hydrolysis studies were carried out on luciferin isolated by both paper electrophoresis and paper chromatography. However,

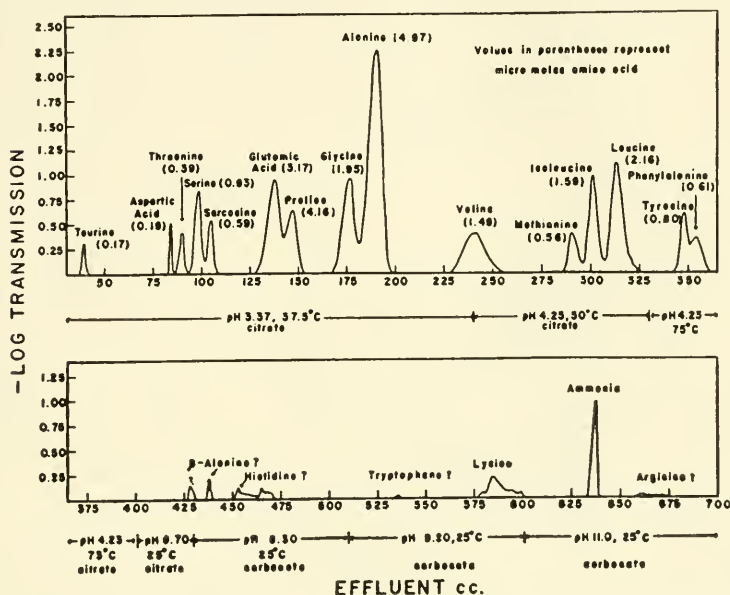


FIG. 6. Isolation of amino acids from luciferin hydrolyzate by chromatography on Dowex 50 column  $0.9 \times 100$  cm according to the method of Moore and Stein (1951). The column was operated in the sodium form. The pH, the buffers used as eluants, and temperature of operation of the column are indicated.

only very small amounts of luciferin (4 mg) were obtainable by these methods and, consequently, composite samples had to be used. The luciferin from these two procedures had, as a solid, a characteristic reddish-orange color but was yellow in methyl alcohol solution. These composite samples did not have the clear-cut ultraviolet absorption bands characteristic of the purest luciferin but, rather, showed some general absorption in the ultraviolet range. Compositing many samples by elution from a large number of paper strips evidently caused some contamination.

Luciferin isolated by paper electrophoresis gave a negative ninhydrin reaction, but a positive test for protein or polypeptide by the method of Lowry *et al.* (1951), using the Folin-Ciocalteu phenol reagent, and the test was accompanied by a loss of luminescent ability. A sample of this luciferin was hydrolyzed by refluxing with 6 N HCl for 16 hours. The hydrolyzate, on evacuating to dryness, gave a very strong ninhydrin test, as did hydrolyzates from two other areas of the

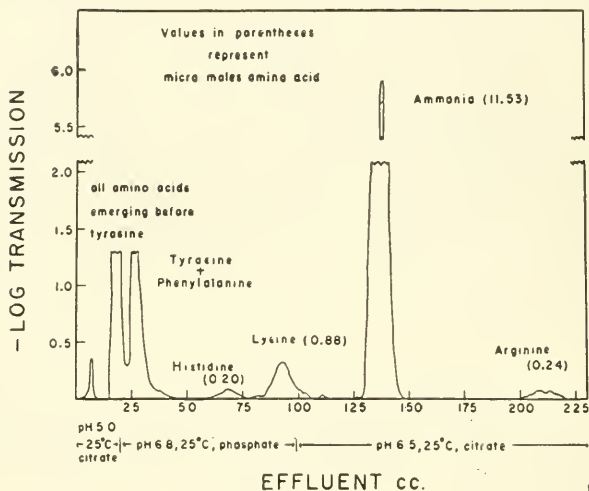


FIG. 7. Isolation of basic amino acids from same luciferin hydrolyzate as shown in Fig. 6. Chromatography was carried out on Dowex 50 column  $0.9 \times 15$  cm according to the method of Moore and Stein (1951). The column was operated in the sodium form. The pH, the buffers used as eluants, and temperature of operation of the column are indicated. The first peak may be taurine, while the second peak represents all the amino acids emerging before tyrosine.

paper, one on each side of the luciferin band. The amount of luciferin isolated by paper electrophoresis was not sufficient to carry out amino acid analysis of the hydrolyzate on Dowex 50 columns.

Luciferin isolated by paper chromatography was also hydrolyzed with 6 N HCl for 16 hours and evacuated to dryness in a vacuum desiccator. The residue gave a very strong ninhydrin test, whereas before hydrolysis only a faint test was obtained. This hydrolyzate also showed some luminescence with luciferase and a characteristic yellow

fluorescence in ultraviolet light. The paper areas on both sides of the luciferin region were also hydrolyzed, both hydrolyzates giving a very strong ninhydrin test. When aliquots of the hydrolyzates from paper chromatography and from paper electrophoresis were repeatedly made alkaline and evacuated each time to dryness in a desiccator, they still gave strong positive ninhydrin tests, indicating that the ninhydrin-positive material was not a volatile substance like ammonia. From the ninhydrin color value, 25% of the luciferin hydrolyzate from paper chromatography could be expressed in terms of equivalence of leucine. Following these tests, the remainder of the hydrolyzate of luciferin from the paper chromatography isolation was analyzed for amino acids in the long Dowex 50 column according to the method of Moore and Stein (1951). The following compounds, listed in approximate order of abundance, were found to be present in the hydrolyzate: either aspartic acid, threonine, or serine; ammonia, arginine, leucine, isoleucine, glutamic acid, alanine, valine, sarcosine, lysine, taurine, and methionine. Of these compounds ammonia and either aspartic acid, threonine or serine were found in very high concentration, although ammonia, arginine, and lysine may be present in higher concentration than indicated. The presence of proline and histidine was uncertain, whereas tyrosine and phenylalanine were absent. There is no doubt but that doubly cycled luciferin contains amino acids. The positive ninhydrin reaction given by hydrolyzates from other regions of the paper chromatogram may have come from oxidized luciferin or polypeptide impurities.

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## DISCUSSION

### The Infrared Spectrum of Anderson's Luciferin

Dr. Mason: In Figs. 8 and 9 are depicted infrared spectra of *Cypridina* luciferin prepared by the method of Anderson. To obtain these spectra dry flakes of amorphous luciferin were placed between rock salt surfaces and absorption was measured by manual adjustment of wavelength setting and null point with a Beckman infrared spectro-

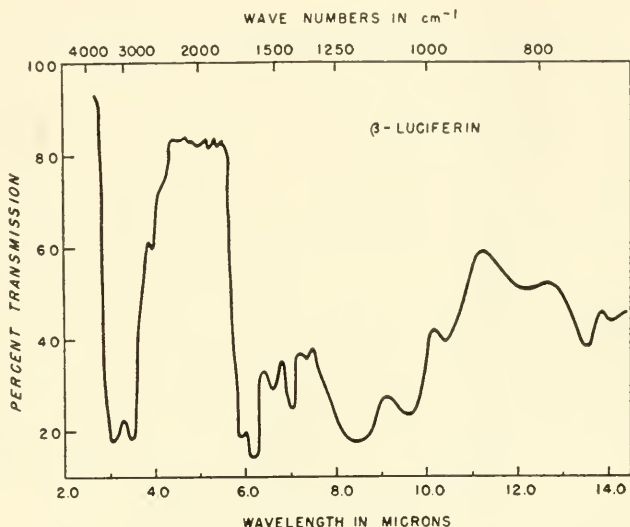


FIG. 8.

meter. Strong bands occur at 3250, 2800, 1680, 1630 and 1510  $\text{cm}^{-1}$ . The absorption of infrared energy in the 2700 to 3100  $\text{cm}^{-1}$  (Fig. 9) region by a luciferin film was measured by Dr. R. C. Gore of the American Cyanimid Company, Stamford. Using a lithium fluoride prism, absorption bands were found at 2860, 2920, 2960 and 3060  $\text{cm}^{-1}$ . It was the comparison of these results with the published spectra of proteins and polypeptides that led to the inference that the peptide bond is present in Anderson's preparations of *Cypridina* luciferin. As it seems evident from Dr. Tsuji's new work that Anderson's luciferin may yet be of a low order of homogeneity, these spectra are being offered as a physical characterization of the material obtained at that stage and as

a basis for comparison with preparations of *Cypridina* luciferin of higher degrees of homogeneity.

In our hands, the amino acids, glutamic acid, glycine, threonine, proline and leucine, isoleucine, or phenylalanine were identified in acid hydrolyzates of *Cypridina* luciferin. Microbiological tests were also performed on luciferin hydrolyzate by Dr. B. D. Davis of the U. S. Public Health Service using a series of amino acid auxotrophes of

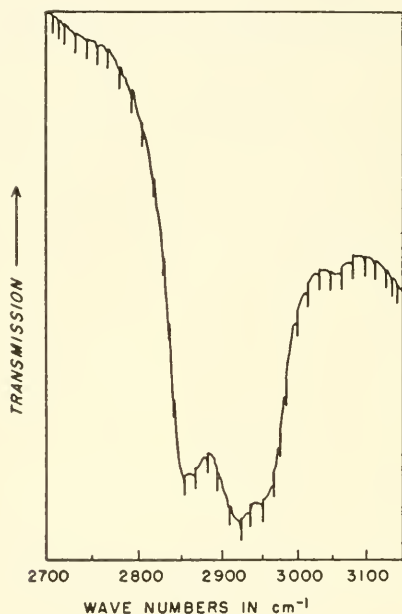


FIG. 9.

*B. coli*; the presence of all the above amino acids was confirmed and, in addition, evidence was found for the presence of methionine, arginine, histidine, valine, and tyrosine.

**Dr. Kauzmann:** Have you tested for the presence of sulfhydryl and disulfide groups in *Cypridina* luciferin?

**Dr. Tsuji:** Sulfhydryl groups do not appear to be present in so far as tests with alkaline nitroprusside and *p*-chloromercuribenzoic acid are concerned. Mason also was not able to detect sulfhydryl groups with alkaline nitroprusside. However, we have observed that when the sulfhydryl blocking agent, *N*-ethyl maleimide, is allowed to stand

with a water solution of luciferin for one-half hour, the yellow color of luciferin is replaced by a pink color and luminescence is markedly decreased when luciferase is now added to the solution. In answer to the second part of your question, we have not tested for disulfide groups specifically.


**Dr. Mason:** Is it possible to estimate the concentration of solute in the solutions with which you measured your absorption spectra and to compare the concentrations with those of the solutions of Anderson's luciferin with which you and Brigham measured your earlier spectra?

**Dr. Chase:** Because luciferin in minute amounts was eluted from filter paper and then finally dissolved in very small volumes of hydrochloric acid for measurement of the absorption spectrum, it was not possible actually to determine the weight of the solute used in the more recent studies. In the measurements of Chase and Brigham (1951), where larger amounts of material were involved, weighing of solid residues was possible and the actual concentration of solute could therefore be determined. It amounted to about 0.02 mg of dry material per milliliter of solution.

Assuming that the absorption spectrum which has been presented really represents luciferin, it is possible, of course, to estimate concentrations from relative extinction values. If this is done, the concentration of solute used in the present measurements would be approximately three times that used in those reported by Chase and Brigham (1951; Fig. 2, p. 532, and Fig. 3, p. 534).







## Biochemistry of Firefly Luminescence

W. D. McELROY and J. W. HASTINGS

McCollum-Pratt Institute and Department of Biology, Johns Hopkins University, Baltimore, Maryland, and Department of Biological Sciences, Northwestern University, Evanston, Illinois

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Judging from the extensive literature on fireflies and glowworms it is apparent that these luminous forms have excited the interest and imagination of scholars and laymen alike. The folklore, history, and scientific accomplishments connected with these luminous forms have been reviewed in detail by Harvey (1952). The first definitive experiment regarding the nature of the components necessary for light production was reported by Dubois in 1885. He found that the luminous organs of *Pyrophorus*, a luminous beetle, would cease to emit light if immersed in hot water. He observed, however, that a cold water extract which had ceased to luminesce could be stimulated to emit light by adding the hot water extract. On the basis of this type of experiment Dubois proposed the theory that there was, in the hot water extract, a substance stable to heat which was destroyed during its luminescent oxidation through the action of a catalyst present in the cold water extract. He named the heat stable material luciferin and the enzyme which catalyzed its oxidation luciferase. This observation which has been greatly extended and clarified by Harvey and associates is the classical luciferin-luciferase test which is routinely made on all new luminous forms discovered by workers in this field.

From a comparative biochemical viewpoint the dissipation of energy in a biological system in units of 40–60 kcal appears quite unique. Most of our information on the generation and transfer of useful energy in biological systems suggests that the process takes

place in a stepwise manner and that each unique step involves energy changes not greater than 10–15 kcal. The two important and obvious exceptions involve either the absorption of radiant energy (photosynthesis) or the emission of light (bioluminescence). In an effort to determine the nature of the oxidative reaction which presumably liberated 40–60 kcal the senior author in 1947 attempted to isolate luciferin from firefly lanterns. The component which was finally isolated as the barium salt was ultimately identified as adenosine triphosphate (McElroy, 1947). Thus by the classical definition ATP was firefly luciferin. Since ATP was known to be the immediate energy source for mechanical activity as well as other processes, this seemed to be a logical extension of the idea of energy transport and utilization in a biological system. Three important questions, however, remained unanswered: (1) Does all the energy for light emission come from phosphate bond energy? (2) What is the importance of the oxidative reaction? (3) What is the nature of the light emitting molecule? Subsequent work on the fireflies indicated that two additional factors were required. One of these turned out to be magnesium ion and the second was a fluorescent substance with an emission spectrum similar to the luminescent spectrum (see Fig. 1). Since this substance is destroyed during luminescence and because of its fluorescent properties, it was called luciferin, the phosphor of fireflies. All subsequent work has indicated that the basic reaction in fireflies can be described by the following reaction:



Both luciferin and luciferase have been highly purified, and the present but brief review will attempt to describe some of the properties of the individual components as well as the entire system.

### Purification of Luciferase and Luciferin

#### Luciferase

The following procedure is based upon the original report of McElroy and Coulombre (1952). Five grams of the dried lanterns of *Photinus pyralis* are ground with sand and extracted three times with a total volume of 100 ml H<sub>2</sub>O. The pH of the extract is adjusted to 8

with NaOH, and the solution is placed in the deep freeze. After freezing and thawing, the inactive precipitate is removed by centrifuging. Twenty-five milliliters of a calcium phosphate gel (16.7 mg/ml) is centrifuged, and the supernatant is discarded. The extract is then thoroughly mixed with the gel and the pH adjusted to 8. After 15 minutes the mixture is centrifuged and the gel is discarded.

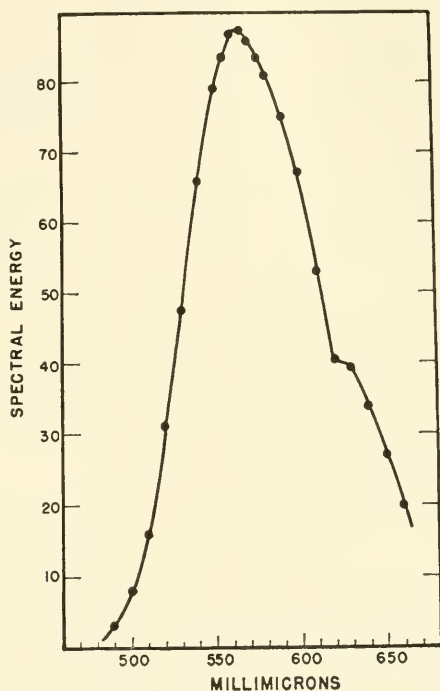


FIG. 1. The emission spectrum of the light emitted from firefly extracts (McElroy and Rainwater, 1948).

The supernatant (prep. II) is considerably more active than the crude extract. Ninety milliliters of the calcium phosphate gel is centrifuged and subsequently mixed with 90 ml of preparation II. The pH is maintained at 8. After 15 minutes the mixture is centrifuged and the supernatant is discarded. In this latter step most of the luciferase was adsorbed onto the gel while the majority of the luciferin remained in the supernatant. To remove the residual luciferin as well as inactive

protein the gel is washed twice with cold alkaline water and then with a 2% solution of  $(\text{NH}_4)_2\text{SO}_4$  at pH 8. Elution of the enzyme is obtained by washing the gel twice with a 7% solution of  $(\text{NH}_4)_2\text{SO}_4$  at pH 8 (prep. III). The final volume of combined eluates of preparation III is 95 ml. Preparation III is then fractionated with  $(\text{NH}_4)_2\text{SO}_4$  in successive steps of 10% saturation up to 50% saturation and then in units of 2 to 3% saturation up to 65%. The pH during this procedure is maintained at 8.0. The major part of the active enzyme is recovered between 57–65%  $(\text{NH}_4)_2\text{SO}_4$  saturation. The latter precipitate is dissolved in 25 ml of water (prep. IV) and the enzyme is readsorbed onto calcium phosphate gel as described above. The supernatant is discarded. The enzyme is eluted with 7%  $(\text{NH}_4)_2\text{SO}_4$  at pH 8 (prep. V) and precipitated by adding solid  $(\text{NH}_4)_2\text{SO}_4$  to 70% saturation (pH 8). The precipitate is dissolved in 5 ml of  $\text{H}_2\text{O}$  and the pH is adjusted to 8 (prep. VI). A further treatment of preparation VI with the low concentration of calcium phosphate gel removes some inert protein (prep. VIII). The activity of the various fractions is summarized in Table I. In this procedure the enzyme was purified approximately 70 times on a protein basis with a total recovery of 15%. In addition, the preparation is completely free of luciferin, and under these conditions no light is emitted upon the addition of ATP.

Preparation VI (Table I) remained stable for several weeks at temperatures below  $0^\circ$ , but was rapidly inactivated at room temperature, especially in dilute solutions. At  $40^\circ$ , approximately 50% loss of activity was encountered in 10 minutes. The preparation was colorless with a sharp absorption peak at 278 millimicrons. The total amount of light which could be obtained from preparation VI was greatly reduced by purification. Within a few minutes after the addition of ATP, the light disappeared completely and reappeared only when additional purified luciferin was added. The results demonstrated that under these conditions a definite, but limited amount of light was obtained for a given amount of luciferin, indicating an irreversible inactivation such as observed in the *Cypridina* system. Although preparation VI responded normally to ATP, no light was emitted with ADP, in contrast to the crude extract. Similarly, ATP which had been treated with hexokinase and glucose failed to initiate light emission in preparation VI. The results demonstrate that the terminal phosphate

TABLE I  
Purification of Firefly Luciferase

Preparation	Light, units/ml-volts	Protein, mg/ml	Specific activity, volts/mg protein
I. Crude	57	10.1	5.7
II. Supernatant 1st $\text{Ca}_3(\text{PO}_4)_2$ gel	166	6.3	26
III. Eluate 2nd $\text{Ca}_3(\text{PO}_4)_2$ gel	80	0.82	98
IV. 57-65% $(\text{NH}_4)_2\text{SO}_4$ precipitate	105	0.60	175
V. Eluate 3rd $\text{Ca}_3(\text{PO}_4)_2$ gel	92	0.33	279
VI. 70% $(\text{NH}_4)_2\text{SO}_4$ precipitate	210	0.627	335
VII. Supernatant 4th $\text{Ca}_3(\text{PO}_4)_2$ gel	182	0.465	391

on ATP is the only immediate labile phosphate group which can be used in the luminescent reaction. These results, as well as others which will be reported later concerning the hexokinase reaction, indicate that an active myokinase had been removed during purification.

### Luciferin

Most of the firefly luciferin remains in the supernatant after the calcium phosphate gel treatment. The supernatant is adjusted to pH 3.5 and extracted twice with an equal volume of redistilled ethyl acetate. All the active luciferin passes into the ethyl acetate. The ethyl acetate is removed by vacuum distillation and the active luciferin is dissolved in a small volume of water. This crude preparation can be used for enzyme assay. Further purification is achieved by adsorbing the luciferin on an acid (2N HCl) treated Dowex 50 column (mesh size less than 80). The column is washed thoroughly with 2N HCl and finally with water. The luciferin is slowly developed on the column by a weak solution of  $\text{NH}_4\text{OH}$  (1.5%). The luciferin migrates down the column in a sharp band and is finally eluted. The luciferin can be readily followed on the column by its brilliant yellow-green fluorescent band. The eluates containing the active luciferin

are again extracted with ethyl acetate, and the latter is concentrated by vacuum distillation. The luciferin is finally concentrated in water. At neutral pH luciferin has two characteristic absorption maxima, one major peak at 330 and a secondary peak at 263  $m\mu$ . The exciting wavelength for fluorescence corresponds to the adsorption peak at 330  $m\mu$ . The concentrated luciferin is slightly yellow in alkaline solution, but it changes to a colorless solution in weak acid. In the former case the fluorescence upon ultraviolet activation is an intense yellow-green, whereas in the latter case the fluorescence changes to a pale

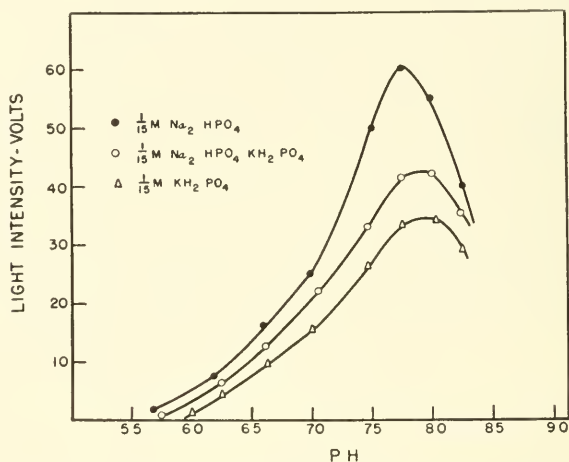


FIG 2. Effect of pH and buffers on firefly luminescence (McElroy and Strehler, 1949).

red. The luciferin can be maintained for several weeks without appreciable loss of activity either frozen in the aqueous solution or in the dried state. In aqueous solution at pH 3.5 and 100° C complete inactivation occurs in 15 minutes, and there is approximately 50% loss of activity in 5 minutes. At pH 10 less than 5% inactivation occurs in 20 minutes at 80° C. The inactive luciferin can be removed from the active by extraction with ethyl acetate at pH 3.5. Under these conditions only the latter is removed from the aqueous phase. The physico-chemical properties of firefly luciferin are discussed in the following paper (Strehler and McElroy).



## Properties of the Luminescent System

*Effect of pH, Temperature, Activators, and Inhibitors.* The luminescent reaction has a rather sharp pH optimum at approximately 8.0 under conditions where none of the other diffusible cofactors are limiting. The results in Fig. 2 indicate that this optimum can be shifted by changing the ionic environment. In a sodium phosphate buffer at pH 7.5 the temperature optimum is approximately 25° C (see Fig. 3) with an experimental energy of activation of 18 kcal.

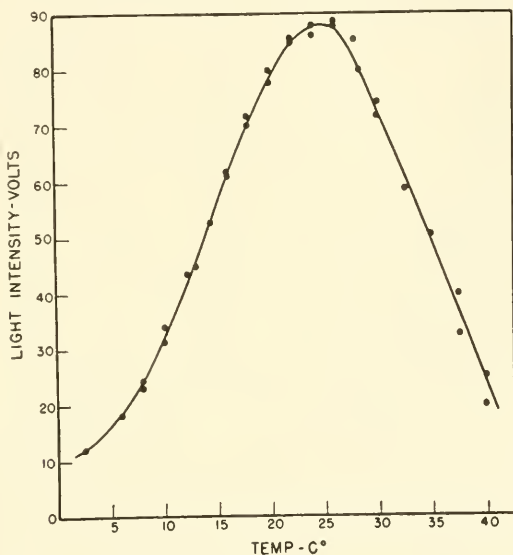


FIG 3. Effect of temperature on firefly luminescence (McElroy and Strehler, 1949).

Although several divalent ions will stimulate the luminescent reaction when crude extracts are used, only Mg and Mn will function in the purified system. In this its behavior seems similar to that of other phosphate transfer systems in which ATP participates. Calcium is a potent inhibitor of the light reaction, competing with the  $Mg^{++}$  ion. The results in Fig. 4 indicate that the flash height as well as the decay to the baseline level is affected by calcium. With a decreased rate of light intensity decay a greater total light output is obtained with

increasing concentrations of calcium ion. It is also significant that the response to inorganic triphosphate is similar to the initial response to ATP with and without calcium present. The results are in keeping with the proposed scheme for luminescence (see below).

The luminescent reaction is essentially insensitive to azide, cyanide, and fluoride although it is strongly inhibited by *p*-chloromercurobenzoic acid. The latter inhibition can be reversed by glutathione. The results indicate a definite involvement of a —SH group for the lu-

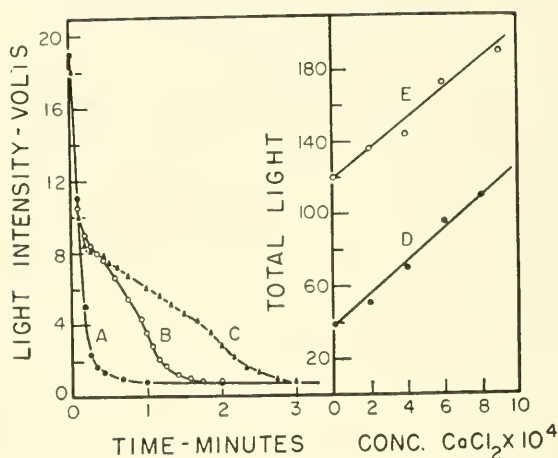


FIG. 4. The effect of calcium on the light intensity and the total light emitted by the purified luminescent system. Curve A, control; B and C contain a final concentration of  $\text{CaCl}_2$  of  $4 \times 10^{-4} M$  and  $6 \times 10^{-4} M$  respectively. Curve E relates to total light emitted when ATP is added initially, while D refers to the secondary response to inorganic triphosphate (McElroy and Coulombre, 1952).

minescent reaction. Inhibitors of the light reaction, which are of particular interest with respect to the structure of luciferin, are those listed in Table II. All compete with firefly luciferin. Of the various substituted compounds 2-phenyl benzothiazole is the most effective. Although 5-methyl- and 5,6-dimethylbenzimidazole are effective competitive inhibitors neither crystalline vitamin  $\text{B}_{12}$  nor 1- $\alpha$ -D-ribofuranosido-5,6-dimethylbenzimidazole influence light emission. Substitution in the one position of the parent compounds completely eliminates inhibitory effects, whereas substitution in the two position potentiates.

The product of the oxidation of luciferin is, likewise, a potent inhibitor of the light reaction. Preparations of oxidized luciferin when added to reduced luciferin completely and apparently irreversibly inhibit light emission. The inhibitory effects can be prevented by adding crude protein preparations to the luciferin mixture before mixing with luciferase. Apparently the protein selectively adsorbs oxidized

TABLE II

COMPOUND	PARENT STRUCTURE	CONC 50 PERCENT INHIBITION MOLAR
1. BENZIMIDAZOLE 5-METHYL — 5,6-DIMETHYL — 1- $\alpha$ -D-RIBOFURANOSID-5,6-DIMETHYL —		$1.9 \times 10^{-3}$ $6.5 \times 10^{-4}$ $2.4 \times 10^{-4}$ NON-INHIBITOR
2. INDOLE 3-METHYL — 1,3-DIMETHYL — ETHOXY — INDOLE ACETIC ACID TRYPTOPHANE		$3.5 \times 10^{-4}$ $4.0 \times 10^{-5}$ NON-INHIBITOR NON-INHIBITOR NON-INHIBITOR NON-INHIBITOR
3. BENZOTHAIAZOLE 2-CHLORO — 2-AMINO — 2-PHENYL — 2-MERCAPTO —		$2.6 \times 10^{-4}$ $4.7 \times 10^{-5}$ $8.0 \times 10^{-5}$ $3.3 \times 10^{-7}$ NON-INHIBITOR
4. BENZOTRIAZOLE		$1.6 \times 10^{-3}$
5. BENZOXAZOLE		$6.7 \times 10^{-4}$

luciferin. Once the inhibition occurs, however, it cannot be reversed by adding the protein to the reaction mixture. Coenzyme A appears to be able to remove the oxidized luciferin from the luciferase since the addition of this cofactor after light initiation will stimulate luminescence (McElroy, unpublished). The secondary response to coenzyme A, under the conditions described, appears to be specific and has been used in the study of CoA synthesis.

### Quantitative Response to ATP and Mg Concentration

One of the interesting characteristics of the crude luminescent system was the rapidity with which ATP was apparently utilized. In crude extracts, the light emission was correlated with the disappearance of ATP and the appearance of inorganic phosphate. When the two equivalents of phosphate from ATP were liberated, the light emission of the preparation ceased. A second addition of ATP restored the luminescence (McElroy, 1947). On the other hand, when ATP was added to any of the partially purified preparations, there

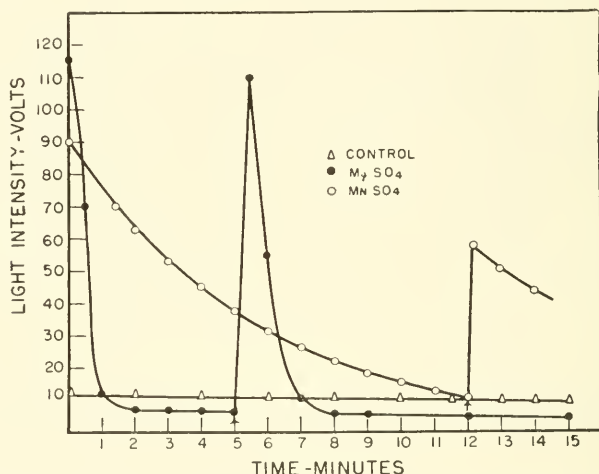


FIG. 5. Light intensity-time relationships for firefly luminescence using partially purified preparations. Secondary addition of ATP at 5 and 12 minutes (McElroy and Strehler, 1949).

was initially a very bright light response, which rapidly decreased to a basal level of approximately 10 to 15% of the maximum (Fig. 5). The low residual luminescence has been observed to last over a period of more than 15 hours. The light intensity of this preparation may be increased by a second addition of ATP. The results definitely indicate that ATP concentration was the limiting factor, as far as light intensity was concerned. Contrary to observations in the crude preparations, however, was the fact that no inorganic phosphate appeared as the ATP was apparently consumed. Several different experiments

were performed in an effort to determine the fate of the labile phosphate groups.

Analysis of samples of the reaction mixture at various times after addition of ATP for labile phosphate established the fact that practically all the labile phosphate groups were still present. Since hexokinase catalyzes the transfer of the terminal phosphate of ATP to glucose, it was possible to determine whether the labile phosphate was still available for this reaction. As shown in Table III, the addition of hexokinase and glucose 5 minutes after ATP caused a rapid decrease in the labile phosphate level. In addition to the evidence cited earlier concerning the presence of myokinase, the results in Table III clearly establish the fact that the enzyme was present, since the phosphate groups of ADP were made available for the phosphorylation of glucose upon the addition of hexokinase.

TABLE III  
Effect of Hexokinase on Labile Phosphate in Luminescent System  
(McElroy, 1951b)  
(Values in micrograms per milliliter)

Time, min.	Reaction A		Reaction B		Reaction C		Reaction D	
	2 ml 0.015 M ATP		2 ml 0.015 M ATP, 1 ml 0.1 M glucose		2 ml 0.015 M ATP, 1 ml 0.1 M glucose, 1 ml hexokinase		1 ml 0.01 M ADP 1 ml 0.1 M glucose 1 ml hexokinase	
	P	$\Delta_7P$	P	$\Delta_7P$	P	$\Delta_7P$	P	$\Delta_7P$
0	8	116	11	126	11	122	5	36
15	10	118			15	78	6	6
30	12	110	10	123	14	45	5	4
45	10	113			14	38		
60	11	111	12	119	16	32		

Fractionation of the luminescent reaction mixture after the addition of ATP with barium acetate showed that over 90% of the labile phosphate was precipitated as the barium salt. This barium-precipitable labile phosphate could also enter into the hexokinase reaction. The results indicate that the ATP was still present in the reaction mixture, even though the light intensity was rapidly decreasing.

The purified enzyme results in Fig. 6 demonstrate that the initial light intensity varies, both with the  $Mg^{++}$  and ATP concentration in a manner suggesting a complex between the two molecules. This is particularly noticeable with low  $Mg^{++}$  concentrations where it is possible to demonstrate an inhibition with high concentrations of ATP. For the intermediate range of concentrations the maximum initial light response is obtained with a ratio of ATP to  $Mg^{++}$  of approxi-

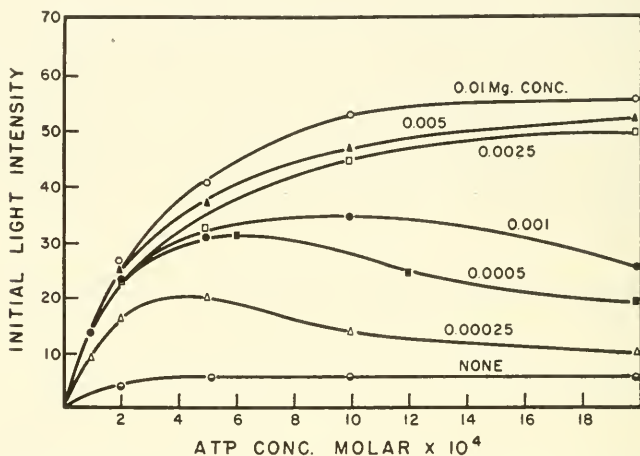


FIG. 6. The effect of varying ATP and  $Mg$  concentration on initial light intensity (McElroy *et al.*, 1953).

mately 1. In addition to the effect of  $Mg^{++}$  on the initial light intensity, there is also a noticeable influence on the decay and final baseline level. The results of such an experiment are presented in Table IV.

In purified enzyme preparations, light emission cannot be elicited by a variety of other phosphorylated compounds which have been tested. The initiation of light emission in the crude extracts by ADP is due to the presence of an effective myokinase. Inosine triphosphate, uridine triphosphate, acetyl phosphate, creatine phosphate, inorganic pyrophosphate, and a variety of other phosphorylated and nonphosphorylated cofactors fail to initiate light. The specificity of response of the firefly extracts has proved useful as a tool for ATP assay in various systems (Strehler and Totter, 1952; McElroy, unpublished).



TABLE IV

Effect of Varying  $Mg^{++}$  Ion Concentration on Light Response to ATP  
(McElroy, 1951a)

$MgSO_4$ concentration, $M \times 10^5$	Initial maximum response to ATP, volts	Basal light intensity at 3 min., volts
0	8.4	4.7
2.0	10.5	3.8
3.5	12.5	3.2
5.0	15.0	2.9
10.0	18.0	3.0
25.0	21	3.0
50.0	25	2.8
100.0	26	2.9

### Light Response to Luciferin Concentration

In the presence of excess ATP and  $Mg^{++}$  ions, the intensity of light from the purified enzyme preparation depends upon the luciferin concentration. Figure 7 illustrates this relationship. The initial maxi-

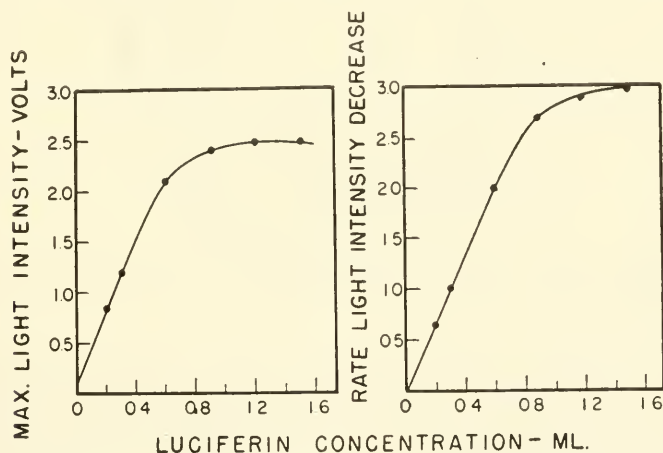


FIG. 7. The effect of luciferin concentration on the initial light intensity and the rate of light intensity decrease (McElroy and Coulombre, 1952).

imum light response is plotted against the luciferin concentration. In these preparations the light intensity rapidly decreases to a low residual level. The rate of decrease of the light intensity, however, is inversely proportional to the luciferin concentration, i.e., at high lucif-

erin concentrations the light intensity decreases faster than at low concentrations of luciferin. This variation in the rate of light intensity decay results in an inverse relationship of light intensity to the luciferin concentration after the reaction has proceeded for 2 to 3 minutes.

The rate of the light intensity decay can be obtained by taking the slope of the straight line which results from plotting the log light

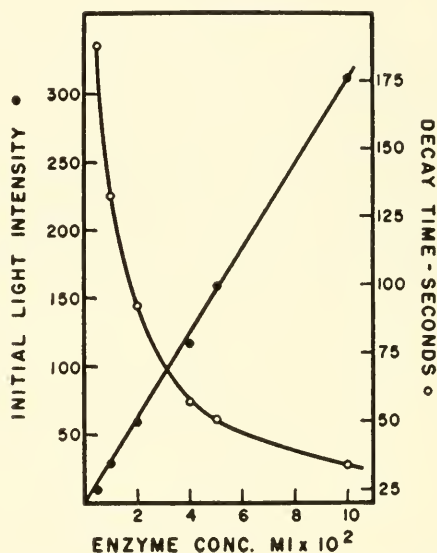


FIG. 8. The effect of luciferase on the initial light intensity and the rate of complexing (McElroy *et al.*, 1953).

intensity versus time for the first 10 seconds of the reaction. By plotting the rates obtained against the corresponding luciferin concentration the relationship shown in Fig. 7 is obtained. The results demonstrate clearly that the rate at which the light intensity decreases depends upon the concentration of luciferin in much the same way as the initial light intensity depends upon this factor. Similar results were reported above for varying concentrations of  $Mg^{++}$  ions, i.e., the steady-state level of luminescence was higher with a low  $Mg^{++}$  ion concentration.

### Light Response to Luciferase Concentration

The results in Fig. 8 indicate that as the enzyme concentration is increased the initial light intensity increases in a linear fashion. Of interest with respect to the decay reaction, however, is the fact that the time to reach the steady-state baseline intensity decreases with increasing concentration of the enzyme. The rapidity of the decay

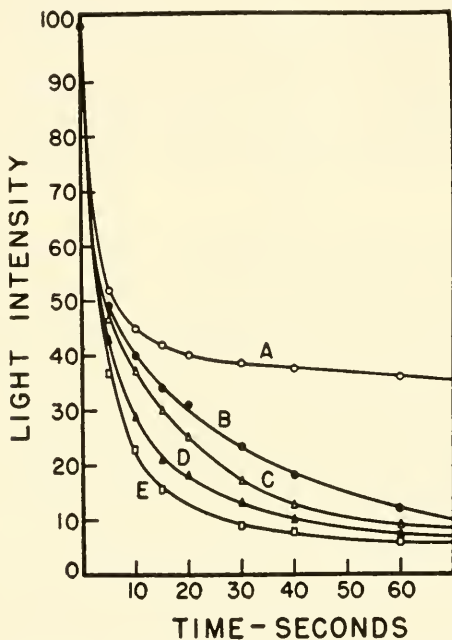


FIG. 9. The effect of pyrophosphatase on the complexing reaction. Purified inorganic pyrophosphatase (10, 20, 40, and 80 micrograms protein) was added to the reaction mixtures (B, C, D, and E respectively) prior to initiating the reaction with ATP (McElroy *et al.*, 1953).

reaction has been observed to vary with different enzyme preparations which suggests that a second factor may be involved. By further purification of the luciferase preparation it is possible to show that a second protein as well as  $Mg^{++}$  are essential for the rapid decay. The most effective protein for accelerating this decay reaction which is present in the firefly lanterns in high concentration is inorganic

pyrophosphatase (McElroy, Coulombre, and Hays, 1951). The results in Fig. 9 illustrate the effect of adding pyrophosphatase to a purified luciferase. As discussed below, the results indicate that luciferase reacts with the second protein to form an inactive complex which effectively removes most of the enzyme from active participation in the light reaction. Although there is some rapid complexing of the luciferase during the first few seconds, it is apparent that the rapid decay to the low light intensity is not observed unless pyrophosphatase is added. The rate of decay to the steady-state level of luminescence is proportional to the added pyrophosphatase. It is not clear why one obtains an initial rapid decrease of the light intensity to approximately 50% of the flash height even in the absence of pyrophosphatase. It may mean that a second luciferase molecule may function in the complexing reaction.

#### Effect of Secondary Addition of Polyphosphates

In preliminary experiments designed to determine whether pyrophosphate would influence the utilization of ATP in the light reaction, it was observed that the addition of the former, after light production by ATP had decreased to a small value, stimulated light production in much the same way as additional ATP (Fig. 10). Since pyrophosphate by itself or pyrophosphate plus adenylic acid failed to initiate light production, it seemed likely that this compound was in some way making the ATP available for light production. A second luminescent response to pyrophosphate occurred in the presence of ADP as well as ATP. With ADP there was a slight lag in the initial light emission in contrast to the instantaneous response to ATP. Furthermore, the maximum light intensity obtained by the addition of pyrophosphate was greater than that obtained with the initial addition of ADP. With ATP the maximum response obtained with a secondary addition of pyrophosphate was approximately 50% of the initial intensity (Fig. 10).

The response of the luminescent system to pyrophosphate was only temporary, as the results in Fig. 10 demonstrate. In many respects it simulates the effect of ATP. The rapid drop in light intensity in the experiments described above is due to a rapid hydrolysis of pyrophosphate by inorganic pyrophosphatase. An analysis for orthophosphate

after the addition of pyrophosphate indicates the presence of a large amount of pyrophosphatase (Fig. 10). The pyrophosphatase has been partially purified by means of  $(\text{NH}_4)_2\text{SO}_4$  fractionation and a number

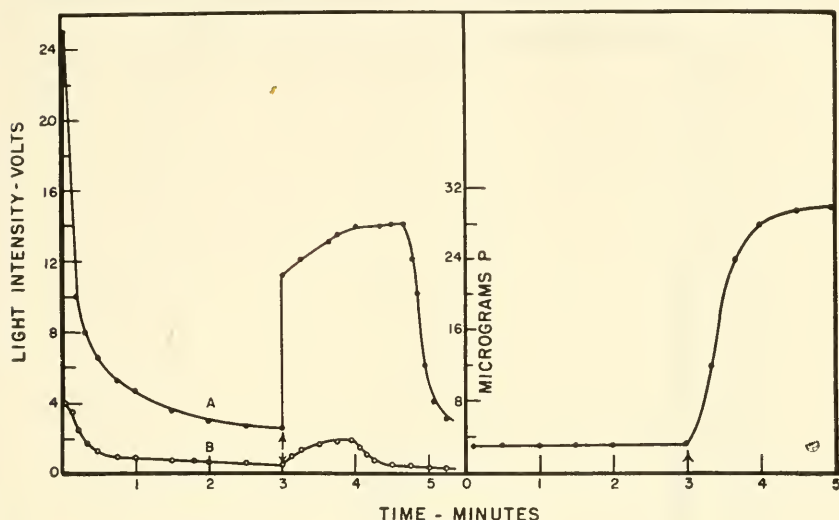


FIG. 10. Effect of ATP and inorganic pyrophosphate on light production. ATP concentration for curves A and B was  $1.5 \times 10^{-4}$  and  $1 \times 10^{-5}$  M respectively. Pyrophosphate (final conc.  $3 \times 10^{-4}$  M) was added at 3 minutes. Phosphate analysis is given in the section at the right (McElroy, 1951a).

TABLE V

Compounds Influencing Utilization of Adenosine Triphosphate  
in Luminescent System

Compound	Final concentration, $M \times 10^4$	Response- % initial
Sodium triphosphate	1.0	63
Sodium metaphosphate	2.0	59
Sodium hexametaphosphate	2.0	51
Sodium pyrophosphate	2.0	50
Thiamine pyrophosphate	2.0	27
Acetyl phosphate	4.0	26
Inosine triphosphate	1.0	24
Glycerol phosphate	30.0	0
Diphosphopyridine nucleotide	10.0	0
Muscle adenylic acid	30.0	0
Hexose diphosphate	10.0	0
Orthophosphate	10.0	0

of its properties studied. The enzyme required specifically  $Mg^{++}$  ion as the activating metal and was inhibited by  $Mn^{++}$ ,  $Ca^{++}$ ,  $Be^{++}$ , and  $Cu^{++}$  (McElroy, Coulombre, and Hays, 1951).

The fact that several compounds, in particular  $Mn^{++}$  and fluoride,

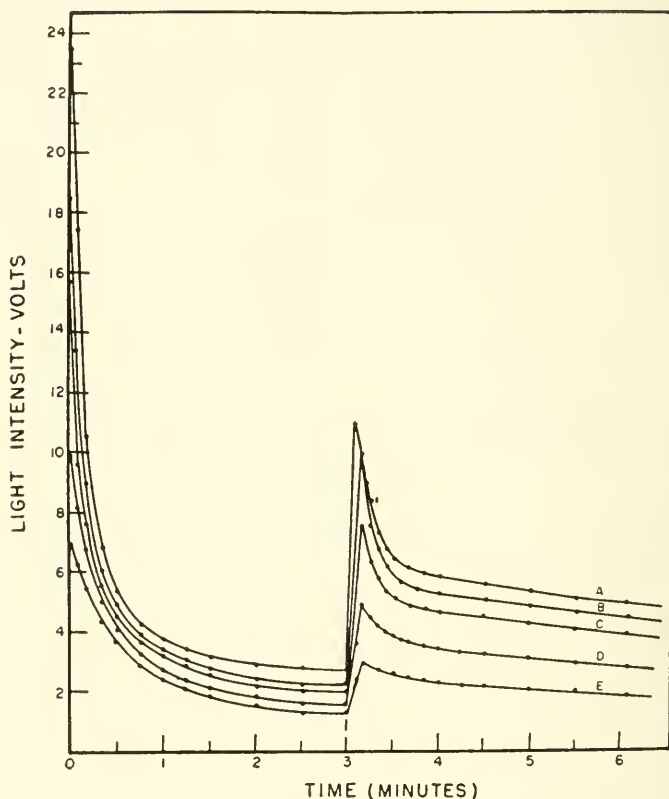


FIG. 11. The response of the luminescent system to varying ATP concentration and a constant ( $5 \times 10^{-5} M$ ) inorganic triphosphate concentration (McElroy, 1951a).

can preferentially inhibit pyrophosphatase activity without affecting luminescence indicates that this enzyme is not essential for light production. The most highly purified luciferase reported above had no pyrophosphatase activity associated with it.

A variety of organic and inorganic phosphate compounds have been tested for their ability to influence light emission in the enzyme prep-



arations after ATP has been added. The results reported in Table V indicate that the most effective compounds are the inorganic meta- and pyrophosphoric acids. It is important that none of the compounds listed will initiate luminescence in the dark preparations.

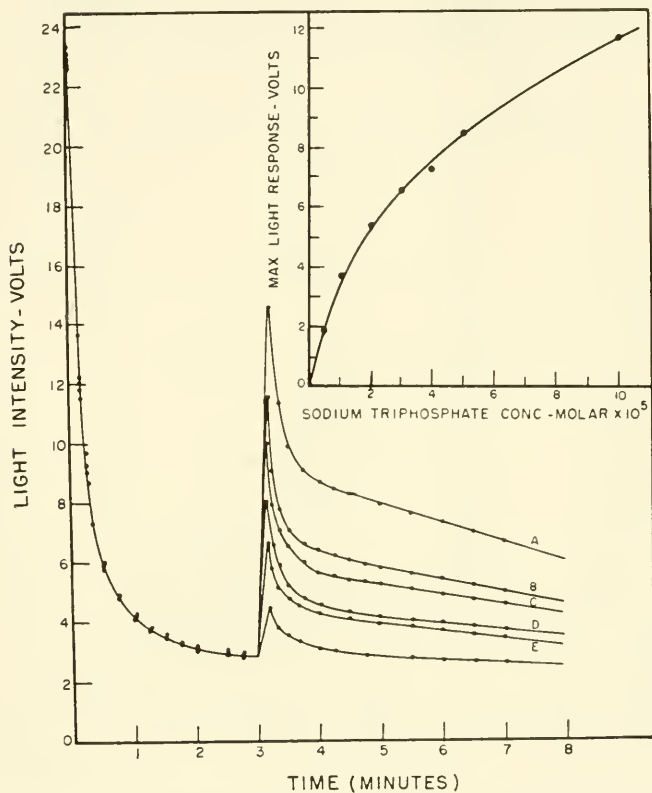


FIG. 12. The response of the luminescent system to varying inorganic triphosphate concentrations and a constant ( $2 \times 10^{-4}$  M) ATP concentration (McElroy, 1951a).

A series of experiments has been performed with varying concentrations of ATP and inorganic triphosphate in an effort to learn more about the kinetics of the stimulation phenomena. The results of these experiments are presented in Figs. 11 and 12. They demonstrate that the initial luminescent response varies with the ATP concentration up to approximately 0.0002 M. The maximum response to a constant

amount of inorganic triphosphate ( $5 \times 10^{-5} M$ ) also varied with ATP concentration. The rate at which the maximum light intensity was attained, as well as its decay when inorganic triphosphate was added, also varied with the initial ATP concentration. The nature of these changes is evident from Fig. 11. A similar picture was obtained when a constant amount of ATP was used ( $0.0002 M$ ) and the inorganic triphosphate concentration varied. The results in Fig. 12 demonstrate

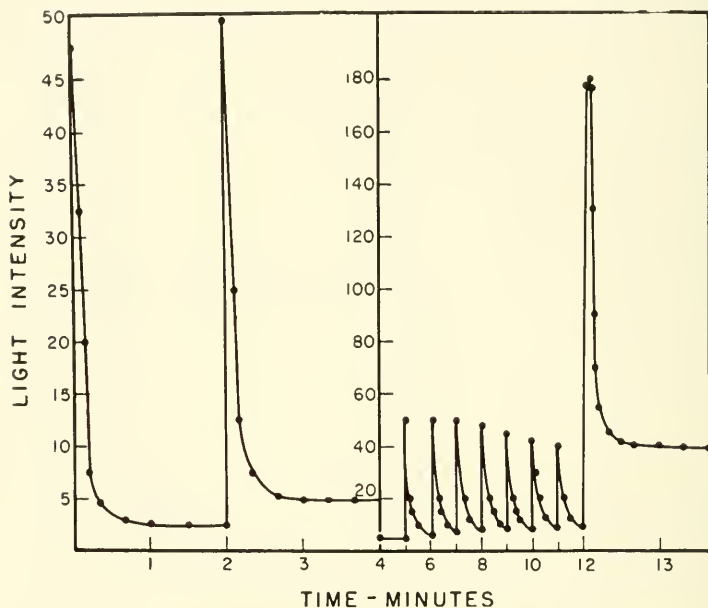


FIG. 13. The luminescent response to the successive addition of luciferase (McElroy *et al.*, 1953).

that the maximum luminescent response as well as the rate varied with the inorganic triphosphate concentration. Higher concentrations were inhibitory when initially added, but with time a certain amount of recovery was obtained, the rate and extent of the recovery depending upon the ATP concentration. It is apparent that the basal level of luminescence attained after adding inorganic triphosphate varied with the concentration of the latter. By adding an excess of ATP it was possible to eliminate the secondary response to inorganic triphosphate, but unfortunately this concentration of ATP inhibited the initial light

intensity. It should be pointed out that no inorganic phosphate was liberated when the inorganic triphosphate was added to the reaction mixture.

### Immobilization of Luciferase—Complexing Reaction

The results reported above have indicated that the rapid depression of light intensity, after the initial addition of ATP, is not due to the utilization of the various substrates of the reaction mixture. The results presented in Fig. 13 are convincing evidence that the luciferase itself is being inhibited or complexed in the reaction. Successive additions of the enzyme elicit responses similar to the initial reaction. With additional enzyme, however, the baseline intensity increases up to approximately the fifth addition. Although additional luciferase

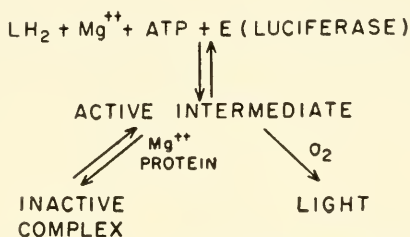


FIG. 14. Scheme for the complexing reaction in firefly luminescence.

gives a flash, the height of the response gradually decreases. The addition of inorganic triphosphate, after nine additions of enzyme, mobilizes a large fraction of the enzyme in the system, resulting in a brilliant flash which is four times the intensity of any of the individual responses.

The decline of luminescence after its initiation with ATP to the low steady-state level is believed to be due to the reversible formation of an inactive complex from an active intermediate (see Fig. 14). This intermediate is presumably composed of luciferase, luciferin, Mg, and ATP. Probably through a series of reactions the active intermediate is finally converted, in the presence of oxygen, to an excited state which subsequently emits light (see below). The low baseline level of luminescence represents, therefore, a steady-state equilibrium between active intermediate and inactive complex. Thus when the

purified components are initially mixed there is, at first, a bright flash of light which then rapidly declines to the low baseline level as inactive complex is formed. Results will be discussed below which indicate that the complexing reaction will occur under anaerobic conditions. Under these circumstances, however, it can be shown that an active intermediate accumulates which rapidly decomposes upon re-admission of oxygen to give a brilliant flash of light. Additional evidence will be discussed which suggests that inorganic pyrophosphate and triphosphate stimulate light production by splitting the inactive complex to form a higher concentration of the active intermediate.

### Effect of Oxygen Tension on the Light Reaction

When a reaction mixture that has reached the basal steady-state intensity is deaerated with purified hydrogen, the light goes out within a minute or two after deaeration is begun (Hastings, McElroy, and Coulombre, 1953). When air or oxygen is readmitted to this solution, an extremely bright flash of light appears with a maximum intensity

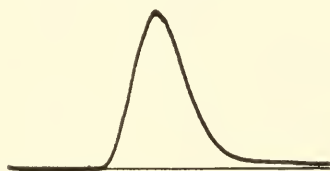


FIG. 15. Photograph of the oscilloscope screen showing the flash reaction. Total time of sweep, 7 seconds (Hastings, McElroy, and Coulombre, 1953).

between 50 and 75 times the steady-state level. The flash is very brief, of about 1 second duration, and quickly returns to the steady-state level. Figure 15 shows an oscilloscope photograph of such a flash. In some cases the curve is symmetrical; in others the rise is somewhat steeper than the fall. The latter cases are attributed to experimental conditions where the oxygen was admitted particularly rapidly. The flash, as observed in these experiments, had a duration of the order of from 1 to 3 seconds. The above procedure may be repeated as often as is desired, using the same reaction mixture, and the same qualitative result is obtained. In practice the luminescence becomes very dim after 10 to 20 minutes, as a result of surface denaturation of

the enzyme through bubbling. The flash phenomenon is interpreted as being due to the accumulation of an active intermediate which, during the steady-state condition under aerobic conditions, is present in only relatively low concentrations. The reaction of the active intermediate with oxygen is an extremely fast reaction for upon the admission of oxygen to such a system this active intermediate is rapidly utilized and gives rise to the flash.

The second method for obtaining data concerning the relation between oxygen concentration and light intensity is as follows. Instead

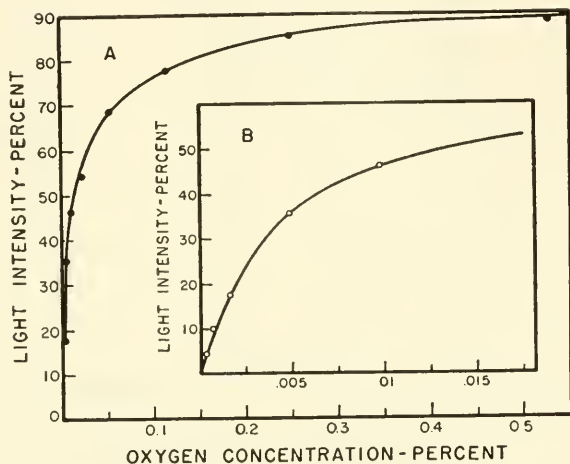


FIG. 16. Relationship between light intensity and oxygen concentration (Hastings, McElroy, and Coulombre, 1953).

of deaerating the system after the steady-state period had been reached, the system was equilibrated with various lowered oxygen concentrations before initiating the reaction with the enzyme (luciferase). The particular gas mixture was bubbled continuously, and the light intensity was measured continuously after the luciferase was added to start the reaction. Under control conditions, i.e., when air is used for the equilibrating gas, the complexing is considered complete within  $2\frac{1}{2}$  minutes from the start of the reaction. In Fig. 16 the intensities observed at this time have been plotted with respect to the oxygen concentrations under which the reaction had been proceeding. When these data are compared with data obtained by the

first method described above, very good agreement is obtained. This fact is regarded as good evidence that the complexing reactions do in fact proceed independently of the oxygen concentration involved. Had the lowered oxygen affected the binding of the enzyme in one way or another, it would be surprising if the two methods for obtaining the relationship between oxygen concentration and light intensity would give the same results.

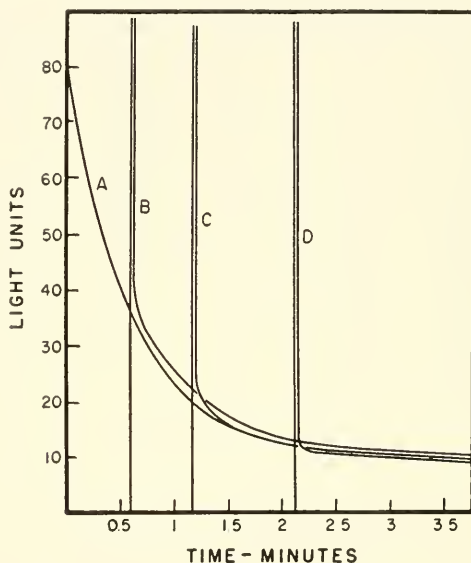


FIG. 17. Effect of mixing reactants anaerobically and adding oxygen at different time intervals. Curve A is a control run in air.

Two additional observations support this conclusion. First, in the above series of experiments air is admitted to the various reaction mixtures after the reading at  $2\frac{1}{2}$  minutes. The typical flash is observed, after which the intensity returns to the low steady-state level. In all cases, irrespective of the oxygen concentration used, the intensity in air returns to a level which corresponds to the intensity of the control reaction in air at the same time after the start of the reaction. Second, if all the components of the reaction are mixed under strictly oxygen-free conditions and air is admitted at various time intervals after mixing, the intensity to which the reaction mix-



ture returns after the flash again corresponds to the intensity level which the control reaction had arrived at in the same time (Fig. 17). If the reactions that lead to the formation of the inactive intermediate did not occur under oxygen-free conditions, one would expect that the reaction would proceed as the control from the time air is first admitted.

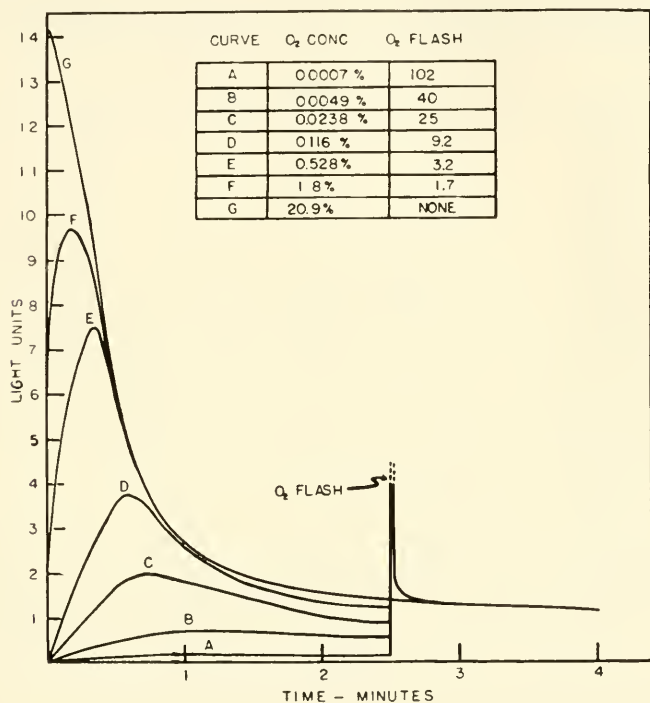


FIG. 18. Effect of various oxygen concentrations on the time course of the light reaction. Air was admitted to obtain the flash height values indicated.

By following the time course of the light reaction at various oxygen tensions it is possible to show that the initial flash intensity is depressed by oxygen tensions which do not influence the steady-state light level (Fig. 18). The results indicate that a rapid utilization of an intermediate by oxygen is limiting in the initial reaction, whereas the formation of this intermediate is limiting after the steady state is reached. In the experiments recorded in Fig. 18, oxygen was admitted

at  $2\frac{1}{2}$  minutes and the resulting flash was recorded. It is evident that the lower oxygen tension leads to a greater flash height. The flash intensity is a maximum when the oxygen tension has been held at approximately 0.001% or less.

### Analysis of Pyrophosphate Action

As indicated in previous sections inorganic pyrophosphatase has a profound influence on the rate of light intensity decrease after the

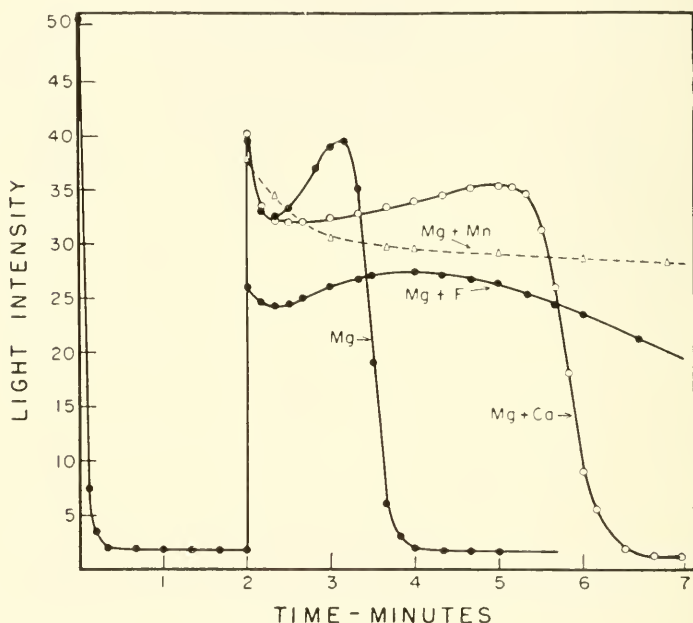


FIG. 19. The effect of inhibitors on the luminescent response to pyrophosphate (McElroy *et al.*, 1953). The pyrophosphate and inhibitors were added at 2 minutes.

reaction is initiated with ATP. Likewise pyrophosphatase greatly affects the response of the light reaction to the secondary addition of inorganic pyrophosphate and triphosphate.

The results in Fig. 19 illustrate one type of response which can be obtained by the secondary addition of pyrophosphate to a partially purified luciferase system which contains pyrophosphatase. Upon addition of pyrophosphate (final conc.  $10^{-4}$  M), there is at first an imme-

diates increase in the light intensity which reaches a maximum within a second. The intensity decreases during the next few seconds to a minimum, then begins to rise, and finally reaches a secondary maximum, from which it rapidly decreases to the low baseline level. Ions that are known to inhibit pyrophosphatase activity greatly influence the response to added pyrophosphate.  $Mn^{++}$ , in the concentration used, should inhibit the action of pyrophosphatase approximately 90%. The results in Fig. 19 indicate that the light intensity in the presence

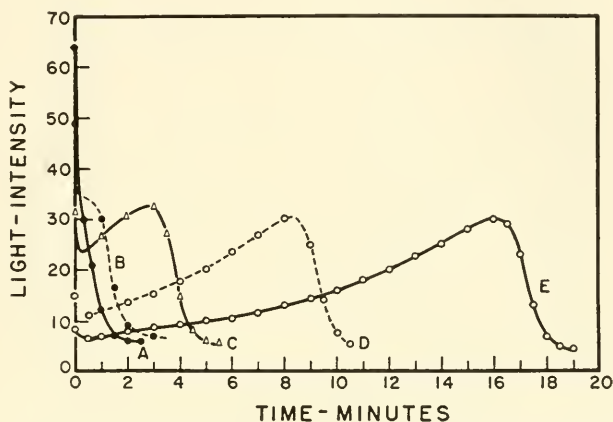


FIG. 20. The effect of pyrophosphate on the luminescent response to ATP (McElroy *et al.*, 1953). Sodium pyrophosphate was added initially to reaction mixtures B, C, D, and E to give a final concentration of  $5 \times 10^{-6}$ ,  $2 \times 10^{-6}$ ,  $5 \times 10^{-6}$  and  $10^{-4}$  respectively.

of  $Mn^{++}$  is maintained at a high steady-state level for considerable time. Intermediate concentrations of  $Ca^{++}$  and fluoride give similar effects.

Pyrophosphate in low concentrations is a potent inhibitor of the luminescent reaction if added prior to the ATP. Apparently it can compete with the latter to form an inactive intermediate with the luciferin-luciferase system. As the results in Fig. 20 indicate, however, this inhibition is slowly reversed if pyrophosphatase is present. The higher the pyrophosphate concentration, the longer is the time required to reach the second peak of luminescence. The quantitative relationships between initial inhibition by various concentrations of

pyrophosphate and the time to reach the secondary peak are shown in Fig. 21.

If inhibitory concentrations of pyrophosphate are incubated with the luciferase preparations containing pyrophosphatase for various time intervals prior to the addition of ATP, a similar recovery is observed, i.e., ATP is not required for this effect. The results of such an experiment are shown in Fig. 22. In curve A the reaction was started in the usual manner with ATP at zero time. After the steady-

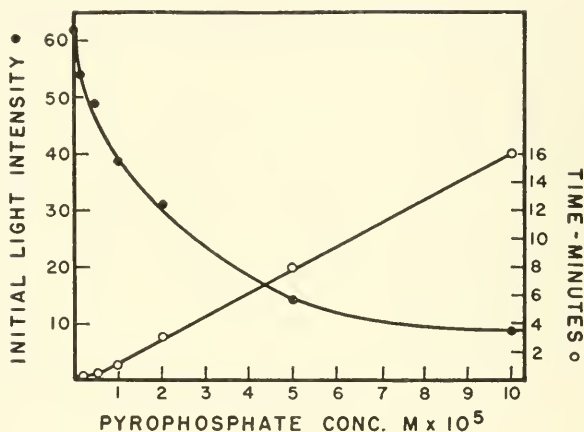


FIG. 21. The relationship between pyrophosphate concentration and light emission (McElroy *et al.*, 1953). The black circles represent initial light intensity while the white circles represent the time required to reach the secondary peak of luminescence as recorded in Fig. 20.

state luminescence had been reached, an inhibitory concentration of pyrophosphate was added. The response is at first a depression followed by an increase in the light intensity, which reaches a maximum 2 minutes later. In the other experiments, ATP was not added initially; however, the pyrophosphate was added at the same relative time after mixing enzyme, buffer, luciferin, and  $Mg^{++}$ . At various intervals after the addition of pyrophosphate, the ATP was introduced to initiate light emission. The results clearly indicate that the longer the incubation time the greater is the initial effect of ATP, in so far as light intensity is concerned. One obtains the secondary peak of luminescence only at certain critical times of incubation. This diphasic

response appears to be directly related to the amount of free and pyrophosphate-bound luciferase, the latter being released by the action of pyrophosphatase. The maximum and normal response to ATP is obtained when the incubation time is extended to a point where all the added pyrophosphate has been hydrolyzed. The phosphate anal-

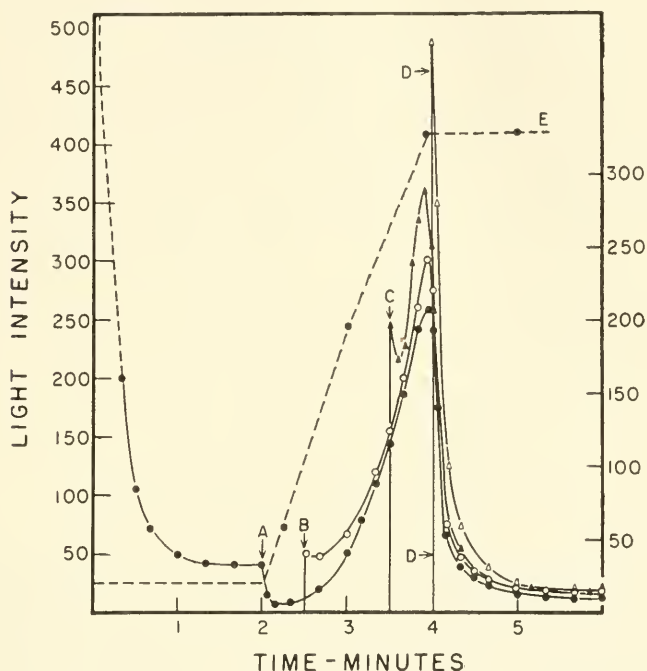


FIG. 22. The effect of delayed addition of ATP on the pyrophosphate response. In curve A, the reaction was started with ATP; 2 minutes later 0.5 ml of 0.01 *M* sodium pyrophosphate was added. In curves B, C, and D, the ATP addition was delayed until 2.5, 3.5, and 4 minutes respectively. Pyrophosphate (0.5 ml of 0.01 *M*), however, was added at 2 minutes to all the reaction mixtures. Curve E represents the average inorganic phosphate concentration in the reaction mixtures.

ysis of the reaction mixture indicates that over 95% of the pyrophosphate is decomposed at the time the secondary peak in luminescence has been reached. The phosphate analysis is also recorded in Fig. 22 and was approximately the same for all the reaction mixtures. It is apparent from this curve that no detectable phosphate is released

from ATP during the rapid complexing reaction, a point that has been established and discussed previously.

If pyrophosphate is added to a highly purified enzyme mixture containing relatively little or no pyrophosphatase activity, the light intensity does not rapidly decrease from the initial flash height to the

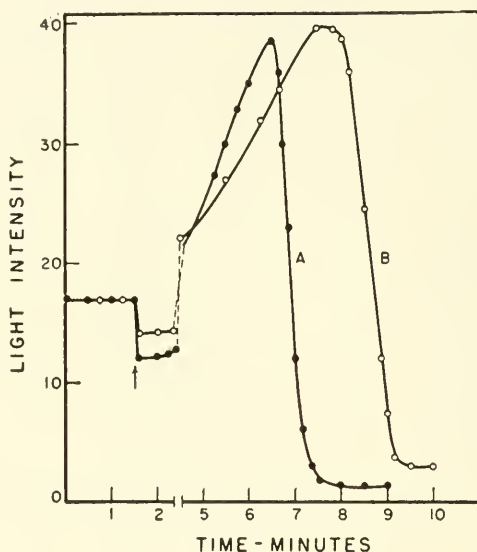


FIG. 23. The effect of delayed addition of pyrophosphatase. The luciferase used was free of pyrophosphatase activity. Pyrophosphate was added initially to give a final concentration of  $5 \times 10^{-4}$  M. The luminescent reaction was allowed to proceed for 5 minutes before the experiment recorded in the graph was started. The arrow indicates when two different concentrations of pyrophosphatase were added to the reaction mixture. To reaction mixtures B and A were added 0.1 and 0.2 ml of partially purified pyrophosphatase (1.0 mg protein/ml) respectively. The initial depression, as well as the steady-state baseline level, after hydrolysis of pyrophosphate, are proportional to the protein added. There was no phosphate liberated during the 6.5 minutes prior to the addition of pyrophosphatase.

low baseline level. The results in Fig. 23 show that the luminescence is maintained at a high steady-state level. Under these conditions no phosphate is liberated, and the secondary rise in light emission is not observed. If, however, a partially purified pyrophosphatase is added, there is at first a depression which varies directly with the amount of protein added, followed by an increase in the light intensity. The rate



of light-intensity increase, as well as the time required to reach the maximum intensity, depends upon the amount of pyrophosphatase added. The curves A and B of Fig. 23 illustrate this point. Phosphate liberation parallels the light-intensity curve in a manner similar to the results presented in Fig. 22. The initial depression of the light intensity, as well as the depression of the steady-state baseline level, by the addition of pyrophosphatase is additional evidence for the importance of a second protein in the complexing reaction. That pyrophosphatase is the important protein for the complexing reaction is indicated by other evidence than was presented above. The ability of a preparation to complex the luciferase parallels the pyrophosphatase activity during purification of the latter. Factors such as temperature and a variety of inhibitors depress the complexing and pyrophosphatase activity in a similar manner.

Inorganic triphosphate is not broken down even by the partially purified luciferase preparations, and it might be expected that a different type of response would be observed with this compound. Several different experiments have supported this viewpoint. In none of these experiments has there been observed the secondary rise after the initial response to added inorganic triphosphate. There is, however, a flash which rapidly decays to a steady-state level. The level of this baseline luminescence is, however, higher with each successive addition of triphosphate, in contrast to pyrophosphate response in the presence of pyrophosphatase. The results of one such experiment are recorded in Fig. 24. Curves A, B, and C represent three successive additions of triphosphate at 2-minute intervals. Although the initial flash response is progressively decreased, the steady-state baseline increases in proportion to the triphosphate concentration. Similar results with different concentrations of triphosphate have been reported previously. Curves 2, 3, and 4 show the effect of successive additions of pyrophosphate to a similar reaction mixture, while curve 1 represents a similar experiment, but with one-half the amount of pyrophosphatase added.

Although the preparation containing pyrophosphatase does not catalyze the hydrolysis of inorganic triphosphate, it influences the luminescent response to the compound. During purification of the luciferase the maximum flash elicited by the secondary addition of tri-

phosphate decreases and may, as in the purest preparations, give no more than 15% of the flash obtained initially with ATP. Addition of the pyrophosphatase to these preparations accelerates the complexing reaction and raises the triphosphate response to as high as 85% of the initial flash. This evidence is additional support for the idea that a second protein is involved in the complexing of luciferase and that both triphosphate and pyrophosphate accelerate the breakdown of this inactive complex. The increase of the baseline level of lumines-

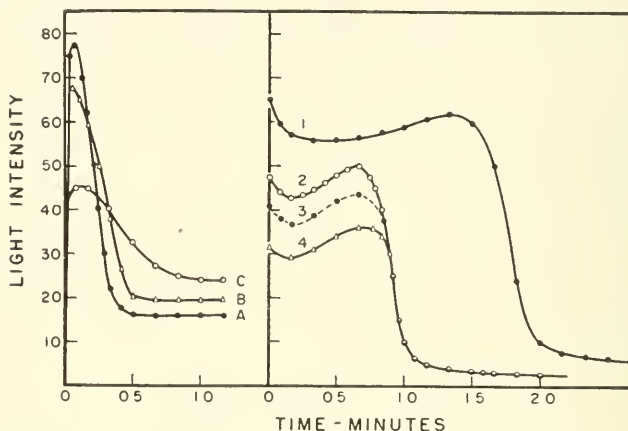


FIG. 24. Light emission with successive additions of triphosphate and pyrophosphate in the presence of pyrophosphatase. In the reaction on the right curves 1,2,3,4 show the effect of successive additions of pyrophosphate. After the first pyrophosphate addition (1) the pyrophosphatase concentration was doubled before additional pyrophosphate was added. Curves A, B, C show the effect of successive additions of triphosphate. The initial response to ATP was 90 light units.

cence with triphosphate is taken as evidence for the complexing of the protein with triphosphate, thus shifting the equilibrium between active and inactive intermediate. Additional protein in this case will restore the original low baseline level of luminescence.

The fact that the triphosphate response cannot be completely eliminated may be due to the fact, as mentioned above, that luciferase itself may serve as the second protein in the complexing reaction.

The results summarized above demonstrate that the rapid decrease of light intensity after ATP addition to firefly extracts is due to the

reversible complexing of the enzyme luciferase. The evidence suggests that an active intermediate composed of luciferin, luciferase,  $Mg^{++}$ , and ATP is first formed, which, in the presence of oxygen, will react to give rise to an excited state that subsequently decomposes to emit a quantum of light. Most of the active intermediate is normally converted into an inactive complex which effectively immobilizes the enzyme. Except for oxygen, all the components necessary for light emission are also essential for the complexing process. The formation of the inactive complex is accelerated by  $Mg^{++}$  and by additional luciferase or other proteins, particularly inorganic pyrophosphatase. The level of the low light intensity appears to be a measure of the steady-state equilibrium between active intermediate and inactive complex. Removal of the inorganic pyrophosphatase by various means affects both the basal light intensity level, as well as the rate of decay.

The relationship between  $Mg^{++}$  and ATP for maximum luminescent activity ( $Mg^{++}$  to ATP ratio of approximately 1) suggests that the true substrate for the formation of the active intermediate with the luciferin-luciferase is a specific  $Mg$ -ATP complex. Similar suggestions have been made by Hers for liver fructokinase and by Kielley and Kielley for mitochondrial adenosinetriphosphatase (ATPase). The relationships between  $Mg$ , inorganic pyrophosphatase, and active intermediate for the formation of the inactive complex indicate a similar type of reaction. The action of inorganic pyrophosphate and triphosphate in stimulating light production, after its initiation by ATP, is attributed to the rapid breakdown of the inactive complex by these agents. In the case of pyrophosphate the evidence shows that it competes with ATP in the formation of the active intermediate. Thus the addition of pyrophosphate before ATP strongly inhibits light emission. The duration and extent of this inhibition depends, however, on the concentration of both pyrophosphate and pyrophosphatase. On the other hand, the delayed addition of pyrophosphate leads to an initial stimulation followed, in many cases, by a decline and then a rise to a secondary peak which rapidly decreases to the baseline level as the pyrophosphate is hydrolyzed by the action of pyrophosphatase. Such results are to be expected if pyrophosphate splits the inactive complex to form initially some active intermediate. The amount of active intermediate formed would depend upon both the nature of the



restore the original steady-state level, it appears that the triphosphate is effectively removing at least some of the protein essential for the complexing reaction.

### Control of Firefly Luminescence

The existence of an inactive complex as proposed above wherein all the components of the system are present without appreciable reaction occurring may be of some significance in considering possible mechanisms for controlling enzyme-catalyzed reactions. The evidence presented suggests that such an inactive complex may be important in controlling the flash of the firefly. Pyrophosphate, which is known to be liberated in several reactions can serve as a trigger in the present reaction after the decay of the initial response to ATP. The pyrophosphatase which is present in high concentrations in the firefly lantern would rapidly decompose the released pyrophosphate, which allows the reformation of the inactive complex leading to the extinction of the light.

The scheme in Fig. 25 illustrates one possible way in which nervous stimulation could lead to the rapid liberation of inorganic pyrophosphate. Since the flash of the firefly is under nervous control this would appear to be a plausible mechanism. It is suggestive that the firefly lanterns contain a very high concentration of coenzyme A.

One cannot fail to be impressed, however, with the striking resemblance between the *in vitro* anaerobic flash and the flash exhibited by the live firefly. As mentioned previously, we believe that anaerobic conditions allow the accumulation of an active intermediate from an inactive one which is rapidly oxidized by molecular oxygen when air is readmitted to give an excited molecule which emits light. Most workers have felt that the firefly regulates flashing by liberating oxygen into the photogenic cells. (See Harvey, 1952, for a review of this literature.) Implied in this statement and in keeping with the fact that "resting" fireflies are not emitting light, is that the photogenic cells are under anaerobic condition. This seems most unusual for insects. Buck (personal communication, see later) has suggested that the firefly must do work in order to remain dark and that only a brief anaerobic period may be essential to bring about the flash response. This suggestion is in keeping with all the biochemical data on the



light reaction. One difficulty remains, however, and that is that even with an excess of inorganic pyrophosphatase the light is never entirely extinguished in the *in vitro* reactions. It is not unlikely, however, that in the intact firefly the low concentration of active intermediate can be rapidly oxidized by components other than molecular oxygen. A brief period of anaerobic conditions may allow the accumulation of an active intermediate to sufficient concentration that it can react significantly with molecular oxygen to give light. Further work on the physiology and biochemistry of the flash reaction will be necessary before a decision can be reached with respect to these various hypotheses.

### Mechanism of Luminescence—Function of ATP

The response of crude extracts of numerous species of fireflies to ATP has now been tested (McElroy and Harvey, 1951). All have given a positive response. In some cases it is necessary to supplement even the crude extracts with additional luciferin before significant amounts of light can be obtained with ATP. It would appear from these experiments that the luciferins of the various firefly species are identical. Additional experimental work is necessary to establish this point definitely. Numerous other luminous forms have been tested for their ability to respond to ATP and thus far positive results have been obtained only with the lymphyrid and elaterid beetles (Haneda and Harvey, 1954). The significance of this fact is not clear, but it may be related directly to the mechanism of control of the light emitting reaction. In *Cypridina*, for example, the luminescent components are liberated from separate glands into the surrounding sea water where they mix, react, and emit light. In other words, the luciferin must be chemically prepared to react immediately without previous dark reactions. This instability in the luciferin molecule is apparent when it is isolated from the dried *Cypridina*. In the presence of oxygen but in the absence of luciferase it rapidly oxidizes without light emission. This is in contrast to firefly luciferin which is relatively stable when purified. Apparently certain dark reactions are essential before it can be oxidized, even in the presence of luciferase. Adenosine triphosphate is essential for this activation process. It now seems likely that firefly luciferin and ATP in the presence of luciferase and




Mg form an active intermediate, possibly a nucleotide, which can now undergo rapid oxidation or peroxidation to form an excited molecule. Whether the ATP contributes, along with the oxidation, activation energy for light emission cannot be answered at present because of the extremely small amounts of the products formed during the reaction. One point which seems clear, both for *Cypridina* and fireflies, is that the luciferins are irreversibly utilized during the light reaction.

Earlier studies on the preparation of crude luciferases from dried and butyl alcohol-treated fireflies suggested the formation of an active intermediate. Some of these preparations would emit light when ground with water but failed to respond when ATP was added. It is possible that this represents an active derivative of luciferin which was preserved under these conditions. Further work will be necessary before a definite conclusion can be made with respect to the function of ATP in the light reaction.

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# Firefly Luciferin

BERNARD L. STREHLER

Department of Biochemistry (Fels Fund)  
University of Chicago, Chicago, Illinois

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In the course of the early work on luminescence of firefly extracts (McElroy and Strehler, 1949) one of the factors which was demonstrated to be necessary for light production, in addition to ATP, magnesium ion, and oxygen, was a compound which we termed "firefly luciferin." Subsequently, some of the properties of this compound were described (Strehler and McElroy, 1949). Further physical and chemical studies were undertaken independently by Dr. McElroy at the Johns Hopkins University and by the author at the Oak Ridge National Laboratory. The results presented here represent work soon to be published jointly with Dr. McElroy (Strehler and McElroy, 1954), and Mr. John Sites (Strehler and Sites, 1954) of the stable isotopes division, Oak Ridge.

The luciferin used in these experiments was isolated by two different procedures, one involving partition chromatography on a Celite column with water as a stationary phase and butanol-chloroform as the moving phase. The other involved chromatography on a Dowex 50 column and adsorption and elution from a fuller's earth column. The materials prepared in these two ways appeared to be identical in both the ultraviolet and infrared regions of the spectrum. Elementary analysis (qualitative) indicated the presence of sulfur and nitrogen and the absence of phosphorus. The material is soluble in polar organic solvents and water at neutral pH and partitions into ether from strongly acid aqueous solution.

### Physical and Chemical Properties

The absorption spectrum of luciferin in solutions of various pH's in the ultraviolet visible region of the spectrum appears in Fig. 1. The shift in absorption was studied as a function of pH as was the fluorescence intensity. The change in fluorescence with pH is illustrated in Fig. 2. From both these measurements it appears that luciferin has a

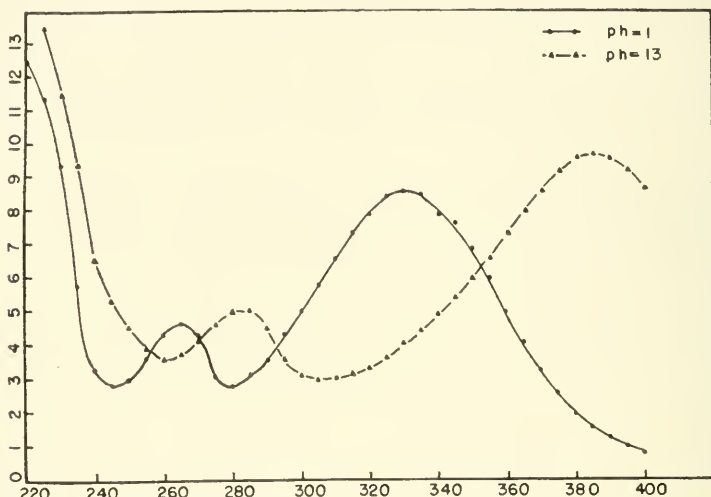


FIG. 1. Ultraviolet absorption spectrum of firefly luciferin at pH 1 and pH 13 in water solution (0.1 *M* HCl and 0.1 *M* NaOH).

$pK_a$  in the neighborhood of 8.5–8.6 (perhaps due to an imine grouping). The infrared absorption spectrum (Fig. 3) of a dry sample of the purified luciferin was determined with a double-beam infrared spectrophotometer (Perkin-Elmer) through the courtesy of Dr. Cameron of the K-25 laboratories in Oak Ridge. Many materials which were considered possible relatives of luciferin on the basis of some of its other physical properties were also examined. Among them were various pteridines, pyrimidines, purines, nucleosides, and nucleotides, as well as riboflavin. Only the riboflavin and luciferin spectra exhibited any marked resemblance from the 2- to 15-micron region. Of particular note is the striking similarity in the absorptions of the two compounds in the 10- to 15-micron region, some bands being

nearly identical, others being slightly displaced from each other (see Fig. 3). These similarities would seem to indicate that luciferin and riboflavin have a predominantly similar general architectural design and that they differ considerably in the nature of the substituents on this framework.

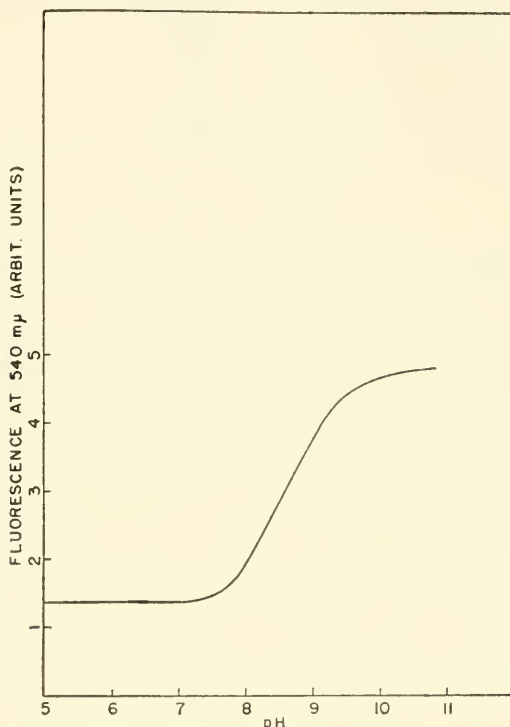


FIG. 2. Fluorescence intensity as a function of pH. The fluorescence was excited with the 365-millimicron Hg line. The pH was varied gradually and pH and fluorescence measured simultaneously.

The electrophoretic mobility of luciferin as a function of pH was determined, and the results are indicated in Fig. 4. Here a  $pK_a$  in the region of pH 3–4 and pH 8–9 are apparent. The more acidic dissociation is probably due to a carboxyl group. In Fig. 5 is illustrated the polarographic half-wave of luciferin. The  $E_0$  suggested by this half-wave is quite far on the reducing side of the hydrogen zero, al-

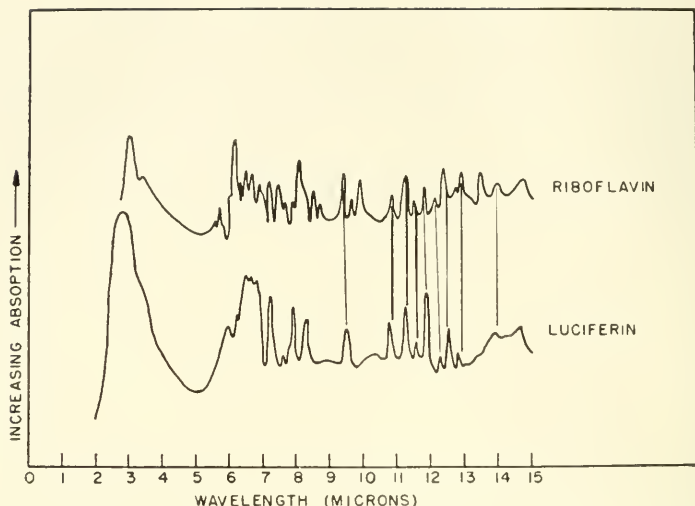


FIG. 3. The infrared absorption spectra of riboflavin and luciferin. Samples were prepared in dry form. In order to compensate for the pronounced scattering, the compensating beam was attenuated. Variations in absorption were thus amplified. On the other hand, this procedure made it impossible to determine the relative extinctions at different wavelengths quantitatively.

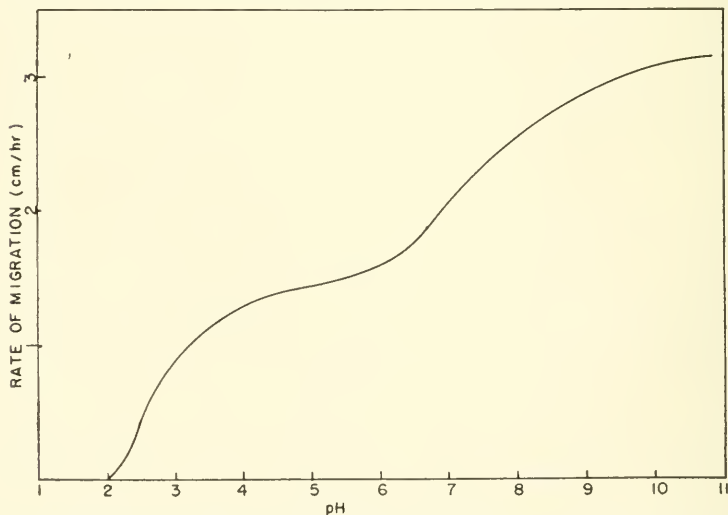


FIG. 4. Migration of luciferin in an electric field as a function of pH. Small T tubes 18 cm in length were filled with ca. 4% agar adjusted to various pH's with phosphate buffer (ca. 0.01 M) in ca. 0.1 M KCl. A luciferin solution was introduced into the middle of the tube through the base of the T with a hypodermic needle. One hundred thirty-five volts were applied and the migration was followed by observing the position of fluorescence.



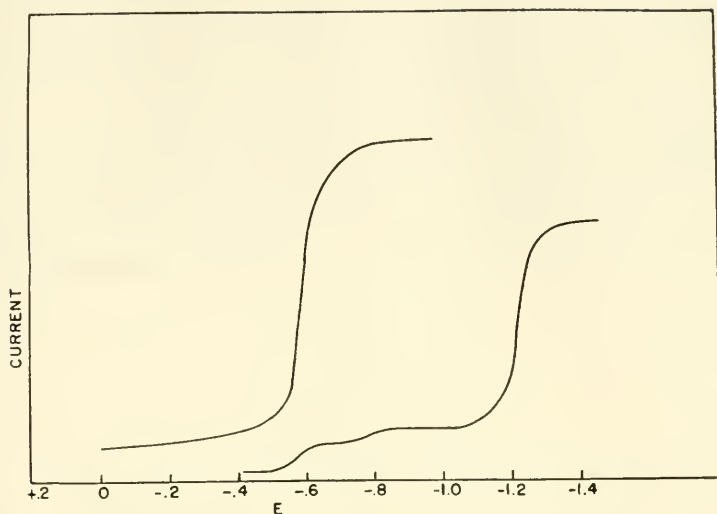


FIG. 5. Polarographic half-waves of firefly luciferin.

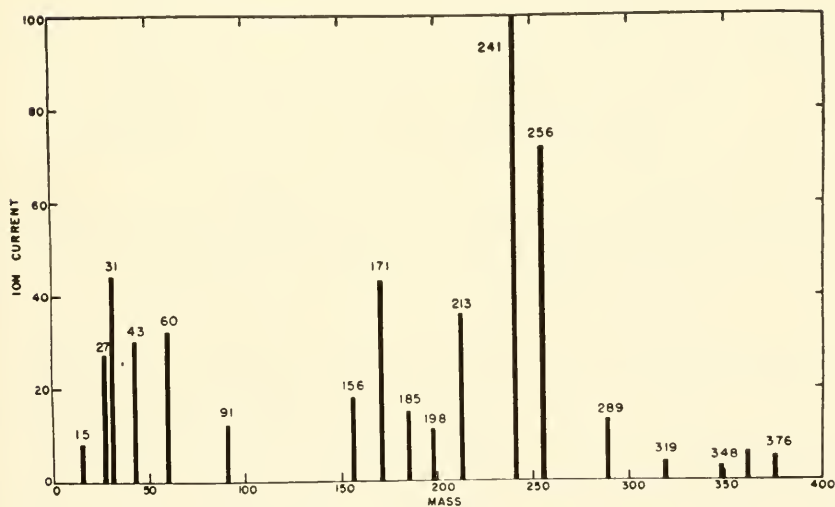


FIG. 6. Mass cracking pattern of riboflavin. The sample of riboflavin (ca. 0.2 mg) was introduced into a special platinum microfurnace consisting of a platinum tube with a narrow slit which was adjusted just below the ionizing beam of the mass spectrograph. The sample could be heated by applying a direct current to the furnace.

though it must be borne in mind that this half-wave may not represent the biologically important one and may, moreover, be considerably modified through association with the enzyme.

Finally, an attempt was made to determine the molecular weight of luciferin through mass spectroscopy. The results of this work clearly yield information on the molecular weight of the compound and also shed some light on the possible structure of this compound. The material was introduced into a special small platinum furnace

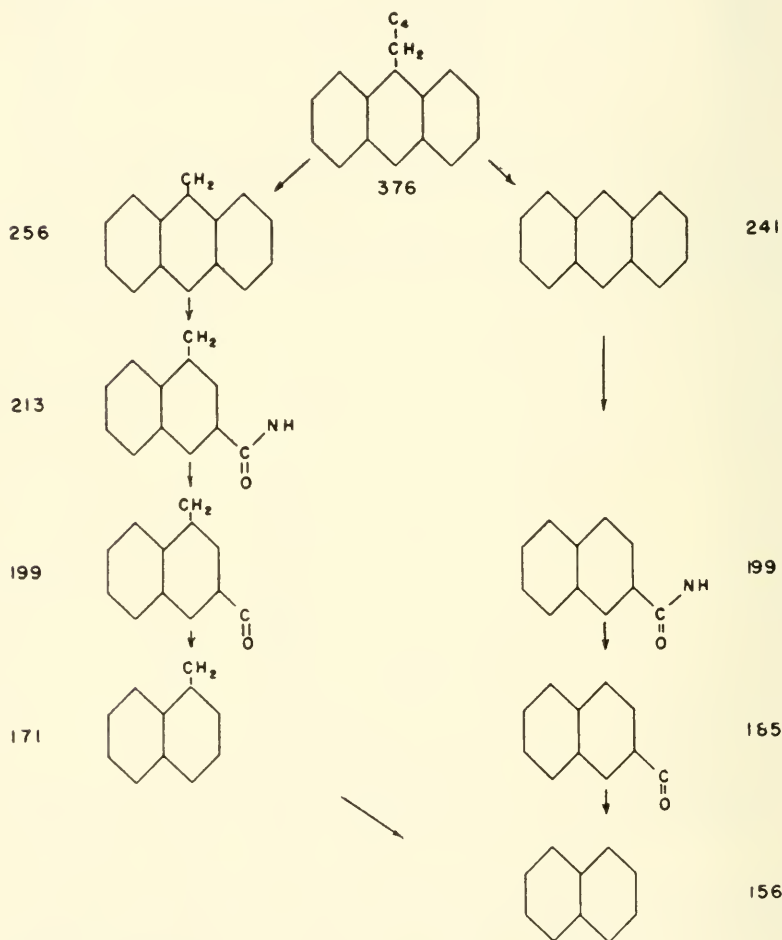


FIG. 7. Structure of proposed fragments arising from riboflavin.

mounted just below the beam of ionizing electrons of the mass spectrograph. Molecules or fragments thereof, volatilized by heating *in vacuo* (within the mass spectrometer) were analyzed both for their mass and the frequency of their occurrence. Riboflavin was run as a model compound and, through analysis of the "mass cracking pattern" of this compound, it was possible to show that the parent compound was successively broken down into smaller pieces in a manner deducible from its structure.

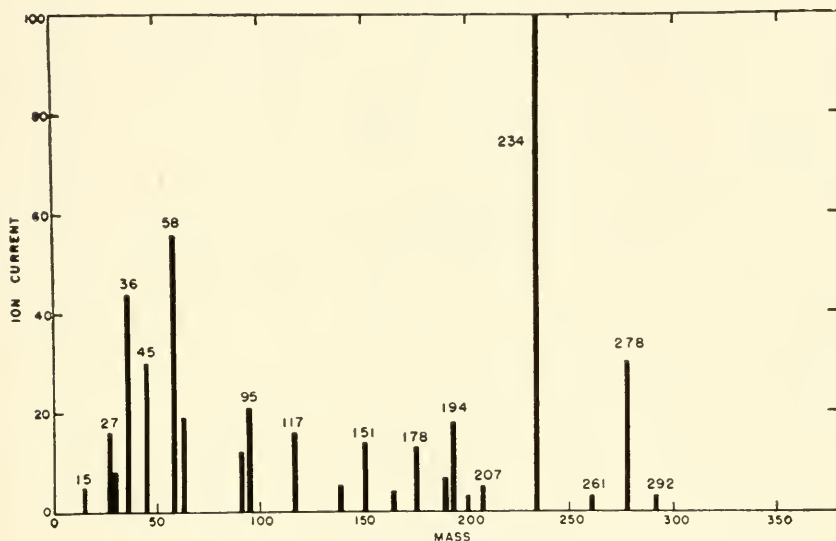


FIG. 8. Mass cracking pattern of firefly luciferin II. The compound used in the mass spectrographic degradation is luciferin II, a compound arising from luciferin through gentle oxidation with  $O_2$  under acid conditions. It bears a strong resemblance to luciferin in physical properties, but has no enzymatic activity.

Figures 6, 7, and 8 show respectively the mass cracking pattern of riboflavin, the structures of the pieces derived from riboflavin pyrolysis, and the mass cracking pattern of luciferin.

In an attempt to untangle the data at hand on the masses arising from luciferin, the mass 234, which seems to be a core of the main molecule, was broken down into all combinations of C, O, H, N, and S which can give mass 234. Some of these combinations are impossible by reason of valence considerations, others are impossible on the basis

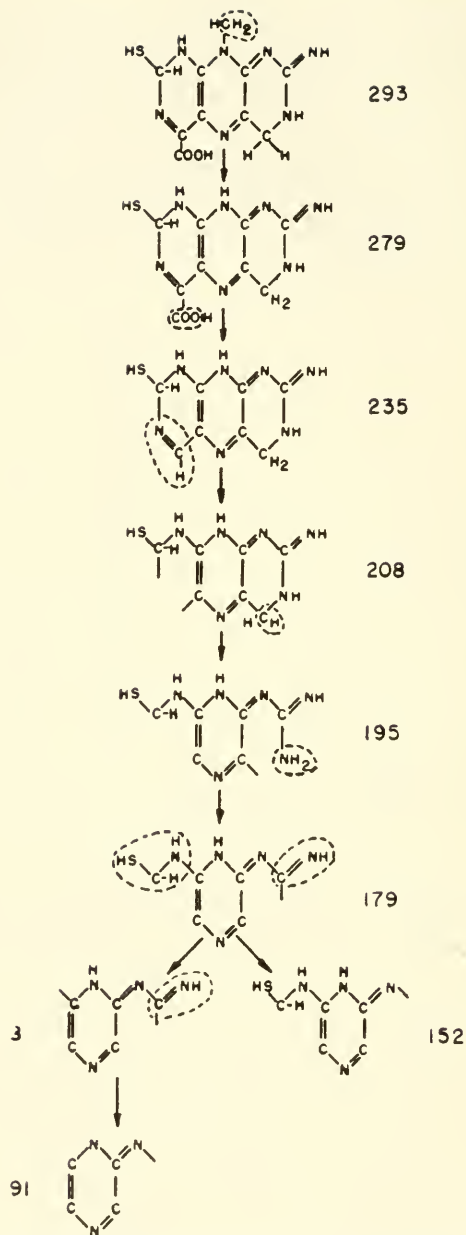


FIG. 9. Proposed structure of firefly luciferin and fragments arising from it through pyrolysis.

of the degree of unsaturation on an empirical basis. An attempt was then made to derive structural formulas consistent with the remaining empirical formulas, taking into account the other physical and chemical evidence available. Although many different combinations and permutations were attempted, only those arising from the dipyrimidopyrazine nucleus could be fitted into the restrictions of the empirical formulas. Subsequently, we synthesized several dipyrimidopyrazines and found, on the part of several of them, physical properties (such as fluorescence and absorption spectra) strongly reminiscent of luciferin, although the precise structure postulated on the basis of the mass cracking pattern was not capable of synthesis by the methods we employed. Figure 9 shows a structure for luciferin consistent with the above data and a proposed scheme of pyrolytic degradation to account for the major mass fragments observed. Note that this cleavage involves the breaking only of single bonds.

It should be emphasized that this structure is perhaps not the only one which could be derived to fit the many properties observed. It is, however, the only one we have been able to formulate which is consistent with all the data available. Note that the calculated masses are always one mass unit higher than the observed masses. If the degradation product is a dimer through the sulfur grouping (disulfide) the rupture of the S—S bond would give an observed mass one less than that calculated.

#### ACKNOWLEDGMENTS

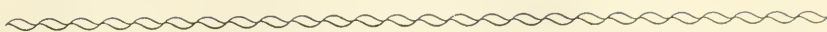
The author wishes to express his appreciation to Dr. Russell Baldock of the Oak Ridge National Laboratory for his generous contribution of time, equipment, and assistance in certain phases of this work, to Mr. John Sites for his collaboration in certain phases, and to Miss E. Brigham and Mr. B. Bottoms for their expert assistance in portions of this work.

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## Factors and Biochemistry of Bacterial Luminescence

BERNARD L. STREHLER<sup>°</sup>

Department of Biochemistry and Institute for Research in Biophysics,  
University of Chicago, Chicago, Illinois

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The blue-green glow emitted by various species of luminous bacteria has been a recurrent object of curiosity among biologists, chemists, and physicists for several scores of years. For workers in each of these fields, the phenomenon of "cold light" emission poses a special set of problems. The biologist is more interested in the evolution, selective advantage, and relation of light production to other functions of organisms; the chemist concerns himself with the mechanism of excitation and the chemical identity of the reacting molecules; while the physicist is primarily interested in the energetics and kinetics of the process and the effect of well-defined environmental variables on light emission. This discussion is an attempt to assess present knowledge of bacterial luminescence touching on all three of these fields. Of necessity the main emphasis here will be placed on the newly available information on the biochemistry of the process, both because this is the least alien to the writer and because it furnishes a starting point for discussion of the other aspects of the problem.

A prodigious amount of work has been expended in studying the effect of various factors on *in vivo* bacterial luminescence, and a cogent summary and analysis of these works is presented in Harvey's *Bioluminescence* (1952). Among the more instructive earlier findings, mainly on salt water species, bearing on this present discussion are the following:

<sup>°</sup> Fels Fund.

1. Bacterial luminescence is a respiratory phenomenon that has an absolute requirement for  $O_2$ , although luminescence is less sensitive to low  $[O_2]$  than respiration (Eymers and van Schouwenburg, 1937; Shoup, 1929).

2. This "light respiration" is essentially cyanide insensitive (Harvey, 1920), although various organic compounds, particularly naphthoquinones, are strongly inhibitory to it (Spruit and Schuiling, 1945; McElroy and Kipnis, 1947).

3. Ultraviolet light inhibits luminescence and shows a discrete "inactivation spectrum" (Gerretsen, 1915).

4. The light emitted is blue-green in color, showing a band with a maximum at ca. 500 millimicrons for a number of species investigated (Spruit-van der Burg, 1950).

5. The yield of luminescence/ $O_2$  consumed is ca. 1/100 to 1/1000 (van Schouwenburg and Eymers, 1936).

6. Luminescence shows a temperature dependence similar to that of many other respiratory processes (Johnson *et al.*, 1942).

7. Pressure-temperature studies indicated that the luminescent system behaves as a typical protein enzyme (Johnson, 1947).

8. Until recently, attempts to extract the system and demonstrate a luciferin-luciferase reaction *in vitro* have been unsuccessful or not capable of confirmation (Harvey, 1952; Gerretsen, 1920; Korr, 1935).

Our success in obtaining brightly luminous extracts (Strehler 1953a), since confirmed in other laboratories (McElroy *et al.*, 1953), must be ascribed to the superior light detecting equipment we employed which enabled us to follow very dim luminescences (Strehler, 1951), quantitatively and for considerable periods, and to the fact that high purity biochemical reagents and intermediates are now available cheaply and in quantity.

Thus, the quantum counter of nearly ultimate sensitivity and low noise level made it possible to measure the dim luminescence exhibited by acetorized extracts of *A. fischeri* while available supplies of DPN, FMN, etc. made it possible to study the effects of these compounds easily and rapidly. The rapidity with which developments have been forthcoming, the general good fortune which attended critical phases of this work, and the complexity of some of the results have made it difficult to keep interpretation abreast of experi-

ment. It is hoped that discussion at this conference will deal critically with those aspects of the work which we have handled unsatisfactorily because of limitations of time and background.

### Extraction of Luminescent System

It had been noted repeatedly (Harvey, 1952; Korr, 1935) that the luminescent powders obtained by a variety of quick-drying methods will emit light when they are suspended in water. Similarly, in unpublished experiments performed in early 1951, Dr. Charles S. Shoup and I were able to obtain light for a few minutes when we added acetone-extracted bacterial powders to water. Addition of boiled extracts did not result in measurable effects once the luminescence had disappeared. However, with the possibility in mind that the oxidant rather than "luciferin" might be limiting, we added hydrogen peroxide to the dark extracts and obtained considerable light. Further studies indicated that one of the compounds responsible for this chemiluminescence in the presence of peroxide is a flavin (Strehler and Shoup, 1953).

Partly because of the press of other work and partly because of our skepticism of the eventual success of further work, these powders remained for nearly two years in a deep freeze before we again attempted to extract the luminescent system. A systematic study was again undertaken in the fall of 1952 with almost immediate success (Strehler, 1953; Strehler and Cormier, 1953). The crucial finding from our point of view was the fact that the duration of luminescence and its restoration by added agents depended on the concentration of powder used. Low concentrations were incapable of luminescing for longer periods or of responding to added biochemical reagents, while a tenfold increase in the ratio of powder to water sustained a continual luminescence and exhibited a "luciferin-luciferase" reaction. This effect is illustrated in Fig. 1 while Fig. 2 illustrates the effect of dilution on the system.

### Nature of Diffusible Requirements

#### DPNH<sub>2</sub> and FMN

Once a sustained and renewable luminescence was attainable a wide variety of shelf biochemical reagents were tested, including

DPN, TPN,  $\text{DPNH}_2$ , riboflavin, FMN, FAD, Co A, lipoic acid, thiamin pyrophosphate, ATP, ADP, AMP, glucose-6-P, glucose, fructose, phosphoglyceric acid,  $\alpha$ -ketoglutarate, acetate, acetyl phosphate, citrate, malate, succinate, lactate, ethanol, acetaldehyde, vitamin A,

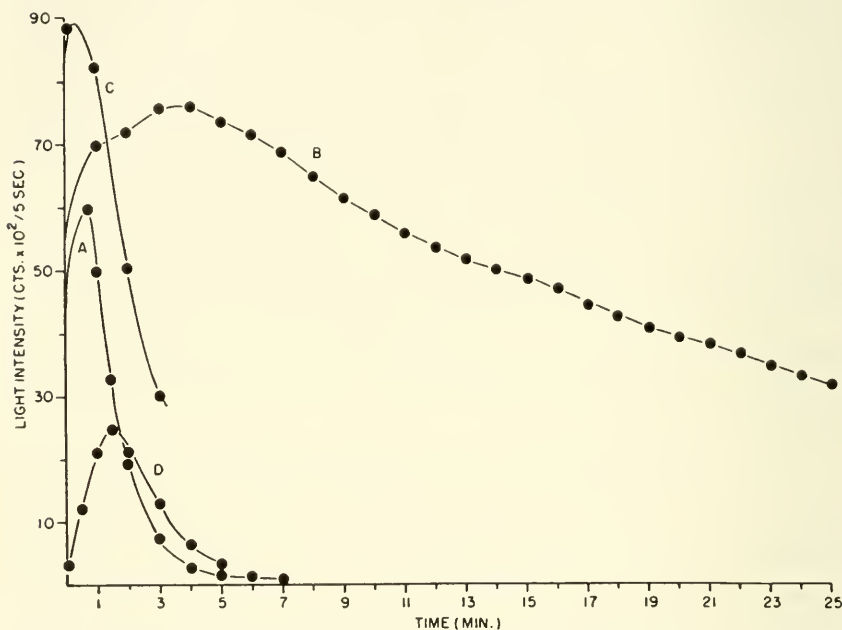


FIG. 1. Time course of luminescence emitted by bacterial extracts. A, 2 mg/ml acetonized bacterial powder added at zero time; B, 20 mg/ml acetonized bacterial powder added at zero time; C, 20  $\mu\text{g/ml}$   $\text{DPNH}_2$  added at zero time; D, 16  $\mu\text{g/ml}$  DPN added at zero time. Water solution, temperature, 23° C.

pyridoxine, ascorbic acid, glutathione, cysteine, glycine, versene, vitamin K, 2-methyl-1, 4-naphthoquinone, vitamin E, benzoquinone, hypoxanthine, xanthine, vitamin D, numerous steroid hormones, hemoglobins, a variety of vitamin-rich concentrates, yeast extract,  $\text{Mg}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Pb}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Co}^{++}$ , etc., and cytochrome c. Most of these were tested not only alone, but in combination with other components.

Of the compounds examined only the following showed marked effects: DPN,  $\text{DPNH}_2$ , riboflavin, FAD, FMN, naphthoquinone, Co A,

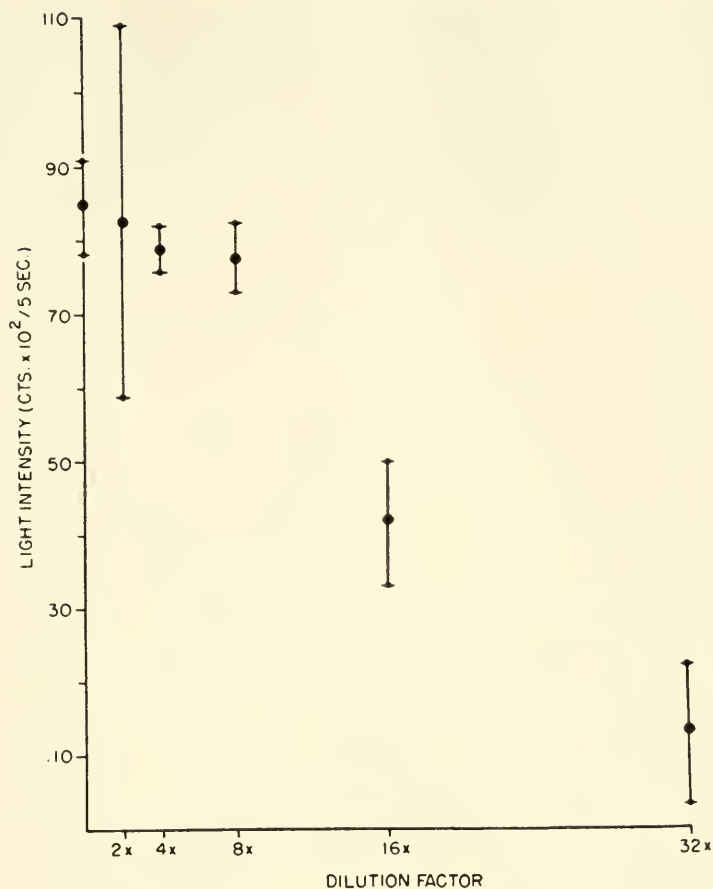


FIG. 2. Effect of dilution of bacterial enzyme extract on luminescence. Each tube contains: 600  $\mu$ g DPNH<sub>2</sub>, 0.2 ml phosphate buffer, 0.01 M, pH 7.0; 0.3 ml of stock extract or of diluted extract (diluted just before measurement). Total volume, 0.6 ml. Stock extract: 13 g acetonized powder in 100 ml of water, centrifuged 1 hour at 0° C on Serval centrifuge at 12,000 rpm. Gross luminescence was multiplied by dilution factor to obtain luminescence per unit weight of enzyme.

malic acid, thiamin pyrophosphate, and  $Mg^{++}$ . Riboflavin, naphthoquinone, and FAD were powerful inhibitors of luminescence, the others either initiating or increasing light production under various conditions.

In the presence of DPNH<sub>2</sub>, only, FMN, of the defined compounds, had a potentiating effect (Strehler and Cormier, 1953). DPN or

DPNH<sub>2</sub> were invariably necessary for luminescence (see Fig. 3). All these studies suggested that the luminescent system is a DPN-coupled respiratory pathway, while the two- to threefold increase obtainable with FMN in the presence of excess DPNH<sub>2</sub> suggested that the oxidase was coupled to oxygen via flavin. That such is indeed the case was clearly demonstrated by the work of McElroy, Hastings, and

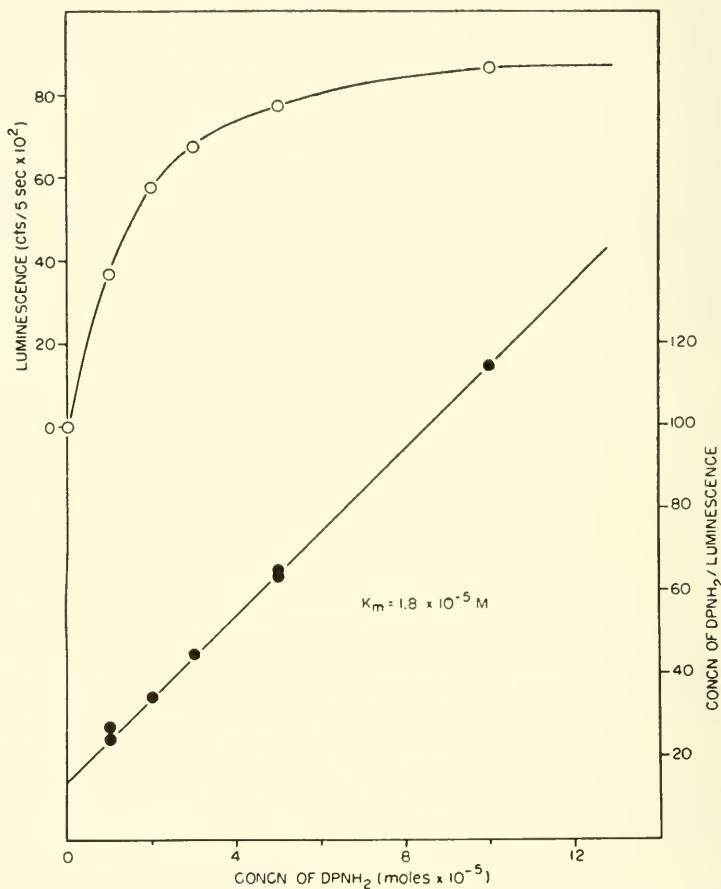


FIG. 3. Effect of DPNH<sub>2</sub> concentration on bacterial extract luminescence: 36  $\mu$ g of palmitic aldehyde plus 4  $\mu$ g of FMN were added to 0.3 ml of an aqueous extract of acetone-powders (4.0 g/100 ml) dissolved in 0.1 M phosphate buffer (pH 7.0); total volume, 3.0 ml. DPNH<sub>2</sub> added as indicated.



co-workers (1953), who were able, through combined acid precipitation and ultraviolet treatment, to obtain a flavin-free system which exhibited luminescence only in the presence of added flavin mononucleotide. The effect of flavin is illustrated in Fig. 4. These workers also reported the preliminary finding of a component in the extracts which they called bacterial luciferin. As is the case with firefly luciferin, this component was destroyed during luminescence.

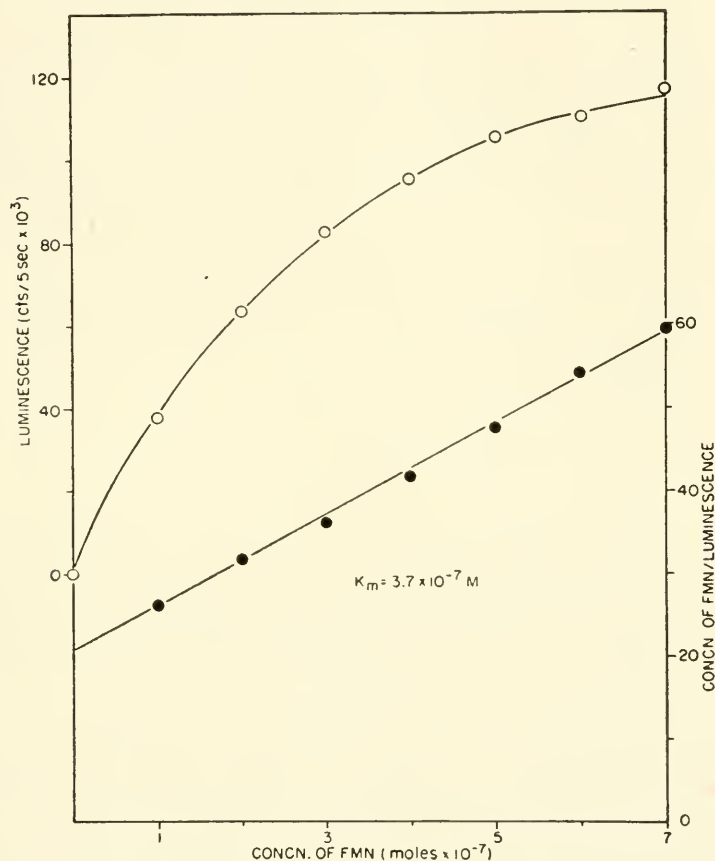


FIG. 4. Effect of FMN concentration on bacterial extract luminescence. To 0.6 ml of a 2% (2 g acetonized powder/100 H<sub>2</sub>O, resolved by precipitation at pH 4-4.5), aqueous extract of *A. fischeri* in 0.1 M phosphate buffer (pH 7.0) was added 500  $\mu$ g of DPNH<sub>2</sub> and 36  $\mu$ g of palmitic aldehyde. Total vol., 2 ml FMN added as indicated.

## KCF (Long-Chain Aldehydes)

We had also noted an increased luminescence when a boiled bacterial extract was added to a system containing excess  $\text{DPNH}_2$  and FMN. (The effect of this factor is shown in Fig. 5.) However, we were never able to separate this material from the heat precipitable

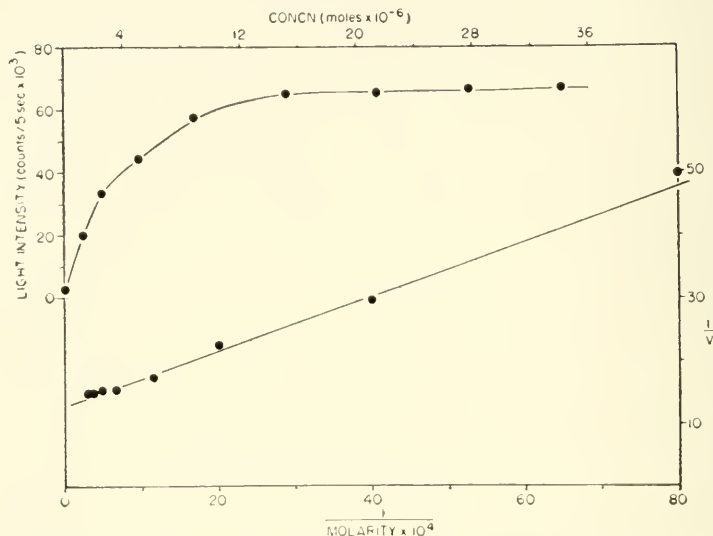


FIG. 5. Effect of aldehyde concentration on *A. fischeri* extract luminescence. 0.1 ml of a 2% aqueous extract of *A. fischeri* was mixed with 1.5 ml of 0.1 M phosphate buffer, pH 7, plus 1000  $\mu\text{g}$  of  $\text{DPNH}_2$  plus 4  $\mu\text{g}$  of FMN, and light intensity was determined. Palmitic aldehyde was then added in concentration indicated and light intensity was measured immediately after each addition.

fractions, although a variety of procedures was employed. Since it seemed possible that some nonspecific effect of the boiled extracts might be involved, we used another source of crude protein, in this case kidney cortex powders, and obtained a large increase in luminescence both from the residue and from the supernatant of this boiled tissue powder (Cormier and Strehler, 1953). We called this component the kidney cortex factor or KCF.

A series of attempts were then made to purify this component by various chemical and physical methods (Strehler and Cormier, 1954).

It was clear that the compound was lipoidal in nature and that it could be purified by partition between organic solvents. Finally, a procedure yielding a material of considerable purity was devised. It consisted essentially of the following steps: (1) extraction of acetonized powders with chloroform, (2) partition between hexane and 5% H<sub>2</sub>O-95% methanol, (3) precipitation of impurities with acetone, (4) precipitation of impurities from small volumes of hexane, chloroform, and methanol, and finally (5) suspension in 1N NaOH and precipitation with HCl.

The material at this point was a yellowish-white sludge, sparingly soluble in water, but relatively more soluble in strong base and organic solvents. Its activity was such that a small fraction of a microgram per milliliter produced a five- to tenfold increase in luminescence.

TABLE I

Comparison of Apparent Dissociation Constants and Maximum Rates of Luminescence in the Presence of Various Long-Chain Aldehydes

Aldehyde	$K_m \times 10^6 M$	Max. Rate (relative)
C <sub>7</sub>	84.0	28
C <sub>8</sub>	20.8	67
C <sub>9</sub>	13.3	59
C <sub>10</sub>	4.1	133
C <sub>11</sub>	2.36	52
C <sub>16</sub>	3.56	81

A variety of qualitative organic tests was applied in order to eliminate certain groups of lipids as possibilities. The nitrogen content was very low (ca. 1-2%), as was phosphorus, while a fuchsin aldehyde test was strongly positive. Treatment of the material with 2,4-dinitrophenylhydrazine gave a good yield of the dinitrophenylhydrazone in crystalline form. Attempts to decompose this derivative and recover activity were unsuccessful. The recrystallized dinitrophenylhydrazone exhibited a sharp melting point at 104-105° C. Since this melting point is in the region ascribed to the derivatives of long-chain fatty aldehydes, we obtained some aldehydes from the stockroom and, although benzaldehyde, acrylaldehyde, and butyraldehyde were not active, synthetic heptaldehyde produced a marked increase in luminescence, one

thirtieth as great on a weight basis as KCF. Finally, KCF was identified as the  $C_{16}$  (palmitic) aldehyde by elementary analysis, mixed melting points of the derivative, and molecular weight determination. All aliphatic aldehydes from  $C_7$  to  $C_{16}$  are active although the lower homologs are relatively less effective. See Table I.

### General Properties of System

#### Color of Light

The spectral distribution of the luminescence of extracts and intact bacteria were compared and found, within experimental error, to be identical (see Fig. 6). This finding along with the other parallelisms between *in vivo* and *in vitro* luminescence makes it likely that the

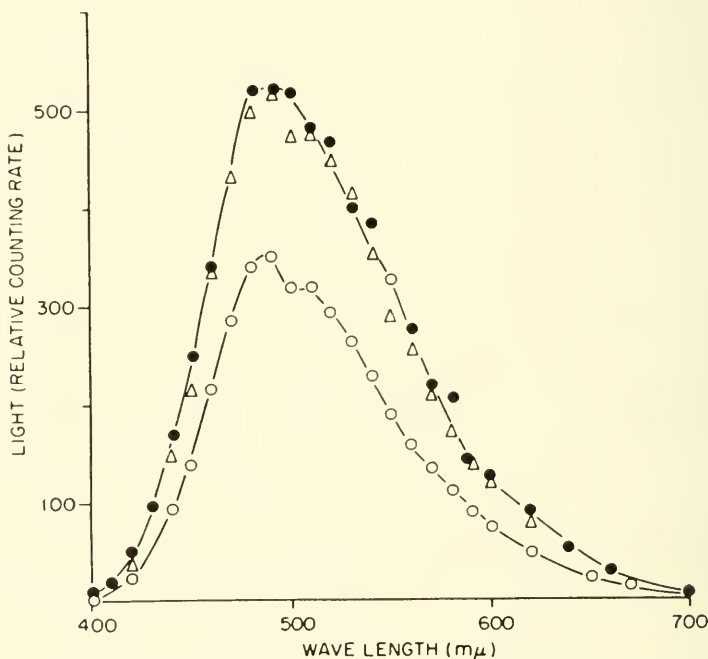


FIG. 6. Emission spectra of intact *A. fischeri* and extracts obtained from *A. fischeri*. 0.5-mm slit width Farrand quartz monochromator. ● Emission of bacteria. ○ Emission of extracts. △ Extract emission normalized to bacterial emission at 490 mμ. The gross reading was not corrected for changes in dispersion of monochromator or sensitivity of photomultiplier.

same reactions are occurring under both conditions. Although it seems probable that flavin in association with luciferase and perhaps long-chain aldehyde, is the light-emitting complex, the considerable difference between the emission maxima exhibited by riboflavin fluorescence and chemiluminescence on the one hand, and the emission of the bacterial extracts on the other, is a puzzling and possibly crucial point at issue.

At least four solutions to this difficulty may be suggested. The first is simply that the flavin is not the emitting molecule, even though the biochemical evidence is fairly convincing that flavin is directly connected with light emission. The second possibility is that the broad flavin fluorescent and chemiluminescent emission is made up of transitions from two different excited states and that only the more energetic of these is formed during the enzymatically catalyzed chemiluminescence. The third alternative is that the binding of the flavin to the enzyme hinders the occurrence of certain vibrational modes, thus largely preventing transitions from the excited state to higher vibrational levels of the ground state and consequently shifting the emission closer to the absorption band. The final possible solution to the difficulty is that the emission in the longer region of the spectrum is quenched by a pigment in the bacteria. Since there is no evidence that such compounds occur in appreciable amounts in luminous bacteria, the process would of necessity involve a mechanism analogous to sensitized fluorescence, with the condition that there is a very low yield of fluorescence from the absorbing entity.

#### Nature of Enzyme—General Properties

The enzyme will not pass through a dialysis membrane, is destroyed by heat, and is nonparticulate (as evidenced by its lack of precipitation under ultracentrifugation). It is sensitive to dilution, inhibited by sulfhydryl inhibitors ( $\text{Hg}^{++}$  and *p*-chloromercuribenzoate), and can be frozen and thawed repeatedly without destroying its activity. It can be separated from some impurities by precipitation with acid, acetone, and  $(\text{NH}_4)_2\text{SO}_4$ . Dr. Green in Dr. McElroy's laboratory is presently engaged in preparing this enzyme in purified form. Whether or not the various activities exhibited by this enzymatic extract in the luminescent sequence of reactions are due to a single component or



to a multiplicity of factors must await its isolation for a definitive answer.

### Effect of Physical and Chemical Environment

The pH dependence of the reaction is illustrated in Fig. 7. The double optima may be due to the semipurified nature of the system,

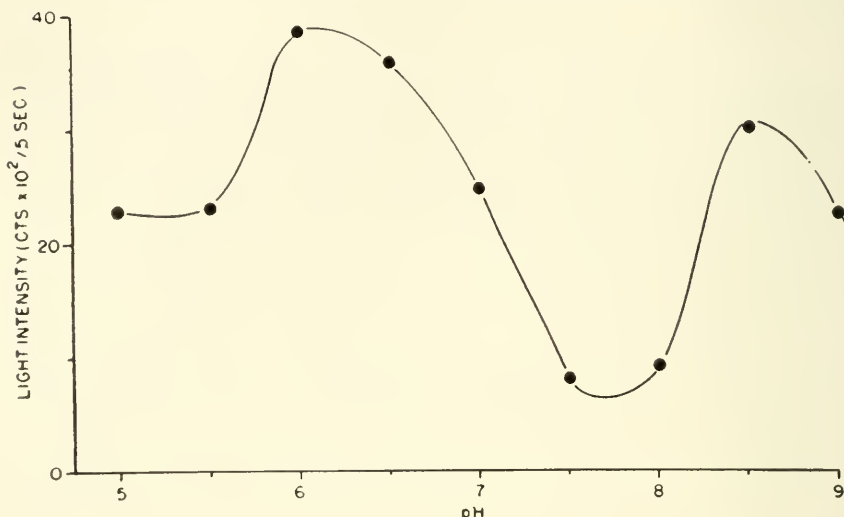


FIG. 7. Effect of pH on dialyzed bacterial extract luminescence: Extract dialyzed for 15 hours against distilled water at 0° C. Each vessel contained 50  $\mu$ g DPNH<sub>2</sub>, 0.2 ml enzyme, 0.2 ml NaH<sub>2</sub> PO<sub>4</sub> (0.01 M) titrated to desired pH with 1 N NaOH; total volume 0.5 ml; temperature 23° C.

but are readily duplicated with crude acetone extracts in the presence of added DPNH<sub>2</sub>.

Temperature profoundly affects the rate of the luminescent reaction *in vitro* and Fig. 8 illustrates a typical result with the crude extract. Particular care must be exercised in making such measurements, since the instantaneous effect of temperature is somewhat different from its delayed effects, even in the lower temperature range. Perhaps some of this effect is due to different pool sizes of intermediates if differential effects are not measured rapidly. The extremely high apparent activation energies (ca. 25 to 31 kcal) observed cast some doubt on the meaning of this measurement, the presence of some arti-



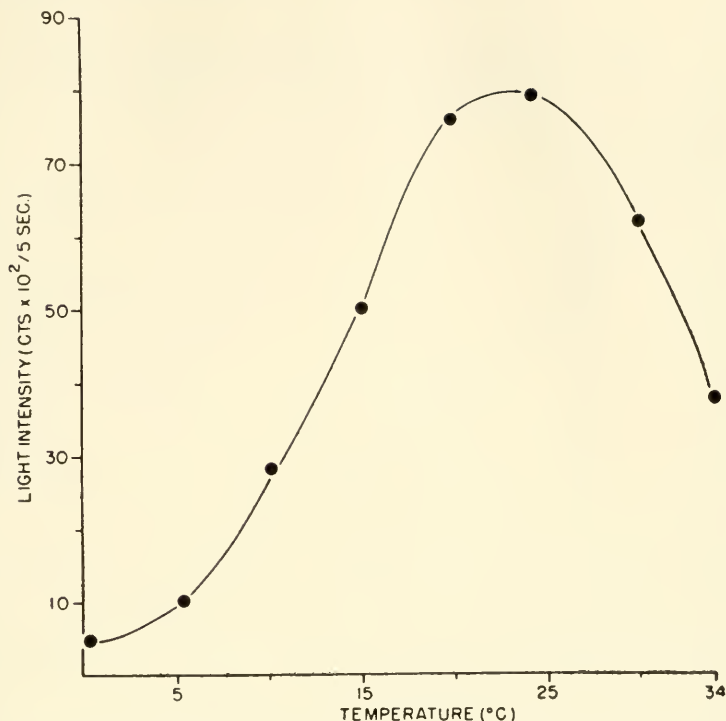


FIG. 8. Effect of temperature on bacterial extract luminescence. 1.0 ml bacterial extract (2%); 1.0 ml phosphate buffer, pH 7.0, 0.01 M; 2.0 mg DPNH<sub>2</sub>. The sample was cooled to 0° C, and its temperature was then raised rapidly with stirring by immersing in hot water (ca. 40° C). Readings were made for 10 seconds at each temperature. Total time for eight determinations, 10 minutes.

fact being indicated, since such a high activation energy would hardly permit the reaction to proceed at significant rates. Possibly a large entropy factor is involved, although another alternative, in such a complicated concatenation of steps as seems to exist, is that nonluminous alternative pathways compete more effectively at lower temperatures.

Various agents have been examined for inhibitory action. Some of those tested are indicated in Table II, while Fig. 9 illustrates the effect of ultraviolet light (Strehler and Cormier, 1953). According to McElroy (private communication), this initial stimulatory effect of 'ultra-

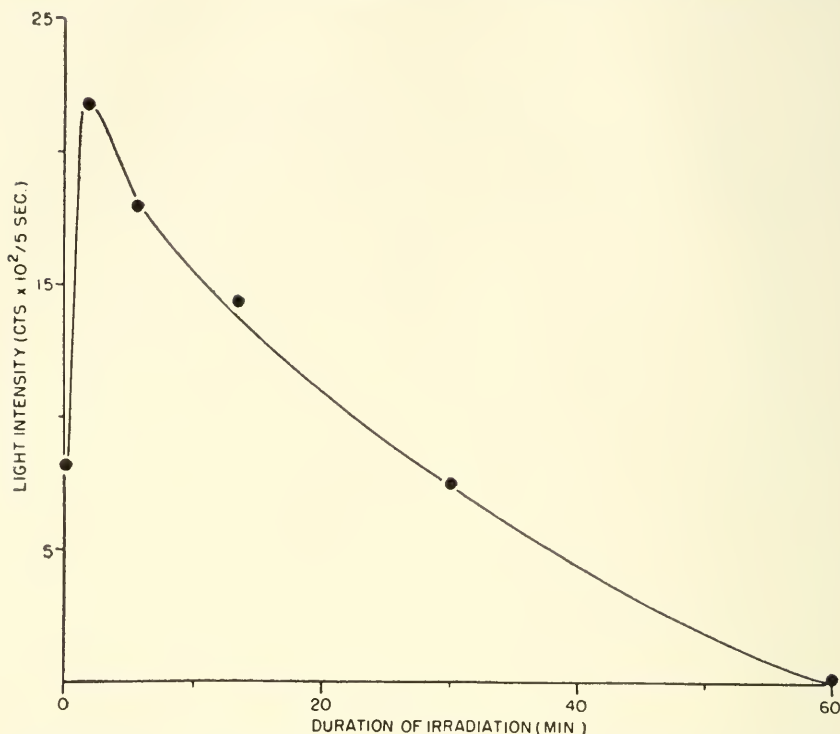


FIG. 9. Effect of ultraviolet illumination on luminescence of bacterial extracts. Two per cent centrifuged extract diluted (1:1) with 0.01 *M* phosphate, pH 7.0, and illuminated with stirring (12 in. distant) by a Keese ultraviolet spot lamp (365  $m\mu$ ). Samples withdrawn at times indicated (0.4 ml) and tested with 300  $\mu$ g of DPNH<sub>2</sub>. Boiled bacterial extract did not restore ability to luminesce to irradiated extracts.

violet may be due to the photo-induced formation of aldehyde. The completely ultraviolet-inactivated system cannot be reactivated in our hands either by addition of the known necessary components or by the addition of boiled active extracts, suggesting that the protein is destroyed under these conditions.

### Comparative Biochemistry

Although little is known concerning the evolution of bioluminescence in luminous bacteria or other luminous forms, it may be possible to draw some tentative conclusions from the comparative bio-

TABLE II  
Effect of Inhibitors on Ultracentrifuged Undialyzed Extracts  
Per Cent Inhibition

Inhibitor	Concentration (molar)	
	$10^{-5}$	$10^{-4}$
KCN	23	47
NaN <sub>3</sub>	30	47
1,2-Naphthoquinone	53	100
2-Methyl-1,4-naphthoquinone	33	100
Iodoacetate	37	26
p-Chloromercuribenzoate	40	71
FAD	46	~
Riboflavin	76	
HgCl <sub>2</sub>	19	73
(Ag) <sub>2</sub> SO <sub>4</sub>	26	100

chemistry of the ten strains of luminous bacteria which have been examined *in vitro* (Cormier and Strehler, 1954). Identical requirements were found for luminescence in terms of diffusible factors among all these strains, e.g., reduced DPN, FMN, and long-chain aldehydes are necessary for the luminescence of their extracts. Certain striking differences were also obvious. These included a wide variation in apparent Michaelis constants and a considerable range of temperature optima and temperature dependences (apparent activation energies) for the luminous reaction.

Inasmuch as there was a great diversity in the morphology of the organisms studied and in the luminescence per unit dry weight of acetone-powders (whose luminescence yield paralleled the *in vivo* brightness of the strains), it would seem unlikely that they are closely related to each other evolutionarily. On the other hand, the identical biochemical requirements for extract luminescence indicate that the mechanism operating in the various strains is probably identical. These differences and similarities can all be accounted for if one assumes that the luminescent pathway in all the luminescent bacteria studied is derived from a normal respiratory pathway (Harvey, 1940). The ability to produce light, then, can be visualized, as arising from a special mutation in a flavin auto-oxidase pathway, permitting the energy liberated on the oxidation of one or more flavins to be lib-

erated in a light-producing reaction. Such mutations, since they probably involve at most only a few steps, could arise repeatedly in unrelated strains. This thesis necessitates no postulation of a special luciferin since flavins, coenzyme I and perhaps aldehydes are ubiquitous among living things.

### Evidence Concerning Mechanism of Interaction of Various Components Required for Bacterial Extract Luminescence

A number of lines of evidence are available which bear on the relationship between the various factors necessary for *in vitro* luminescence. These studies are concerned with kinetic measurements (Strehler and Cormier, 1954b) with the effect of various added components on the respiratory rate (Strehler and Cormier, 1954a), with the effect of reduced flavins on the luminescence with and without added aldehyde (Strehler *et al.*, 1954) and some pressure effects examined in cooperation with Dr. Frank Johnson of Princeton University (Strehler and Johnson, 1954).

#### Respiration

Attempts to determine an effect of long-chain aldehydes on respiration were at first unsuccessful, perhaps because the limiting reaction either in the presence or absence of aldehyde is the  $\text{DPNH}_2\text{-FMN}$  reaction. However, it was possible, by varying the concentration of oxygen in the medium, to show that the aldehyde did in fact affect the level of luminescence and in a parallel manner the rate of respiration at low oxygen tensions. The results of such studies are indicated in the accompanying Fig. 10. From this figure it can be seen that the luminescence as well as respiration—not shown) is accelerated at a low oxygen tension, if aldehyde is added. The presence of aldehyde changes the apparent Michaelis constants for oxygen (both for luminescence and respiration) by about a factor of 4.

#### "Rise Time" Experiments

Another type of kinetic study which was useful in determining the relationships of the various components was the so-called  $\frac{1}{2}$  rise-time experiment based on similar studies with *Cypridina* extracts and intact bacteria reported by Chance *et al.* (1940). The general design

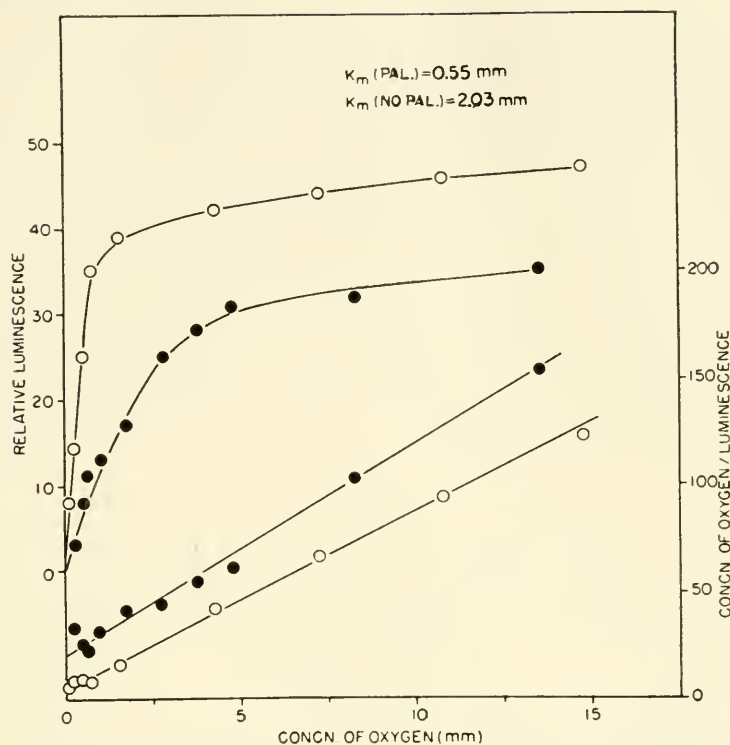


FIG. 10. Effect of oxygen concentration on luminescence of bacterial extracts in the presence and absence of palmital. To 1.0 ml of a 10% aqueous extract of *A. fischeri* in 0.1 M phosphate buffer (pH 7.0) was added 2 mg of  $\text{DPNH}_2$  and 8  $\mu\text{g}$  of FMN. Oxygen tension was measured with a dropping mercury electrode simultaneously with the light emission. 60  $\mu\text{g}$  of palmitic aldehyde was then added to the same aliquot of extract and oxygen tension and luminescence were again measured. Total volume 4.0 ml. ● No palmital. ○ with palmital.

of these present experiments was to add all except one of the components necessary for a bright luminescence to the enzyme and then, at zero time, to add the remaining necessary component. The light output as a function of time after mixing was measured and recorded electronically and the approach to steady-state luminescence was plotted in such a manner as to give an index of the  $\frac{1}{2}$  rise time, i.e., the time required for the luminescence to become half maximal after mixing the various components. It was found that the terminal addi-

tion of factors which, on the basis of the effect of the aldehydes on respiratory activity or other work with nonluminous organisms, might be expected to react with each other, resulted in rise times of very similar or identical magnitudes (Strehler and Cormier, 1954a). For example, if malic acid were used as a hydrogen donor to reduce DPN and the time course of the luminescence was plotted according to the equation  $\log (R \text{ max.} - R \text{ obs.})/\text{time} = -a$ , the slope of the line obtained was the same whether malate or DPN was the last added component. Similarly, oxidized flavin or reduced DPN when added last exhibited similar rise times, and reduced flavin, oxygen, or aldehyde produced increases in luminescence, whose rate of approach to steady state conditions were comparatively close to each other. These results are summarized in Table III.

TABLE III

Time Required for Half-Maximal Luminescence in Bacterial Extracts  
When Various Essential Components Are Added Last

Component Added Last	Factors Present with Enzyme	Half-Rise Time
O <sub>2</sub>	FMN, DPNH <sub>2</sub> , KCF	0.05
KCF	O <sub>2</sub> , FMN, DPNH <sub>2</sub>	0.08
RFH <sub>2</sub>	O <sub>2</sub> , KCF	0.14
RFH <sub>2</sub>	O <sub>2</sub>	0.27
FMNH <sub>2</sub>	O <sub>2</sub> , KFC	0.075
FMNH <sub>2</sub>	O <sub>2</sub>	0.41
FMN	O <sub>2</sub> , DPNH <sub>2</sub> , KCF	2.7
DPNH <sub>2</sub>	O <sub>2</sub> , FMN, KCF	2.7
DPN	O <sub>2</sub> , Malate, FMN, KCF	108.
Malate	O <sub>2</sub> , DPN, FMN, KCF	108.

In any system consisting of a number of consecutive steps, it might be expected that the steps further separated from the final reaction would require longer to reach steady-state rates than those separated by one or a few steps. This expectation is borne out in the data presented in Fig. 11.

#### Studies with Reduced Flavins

In cooperation with Dr. Harvey and Mr. Chang of Princeton University a number of experiments were performed to test the hypothesis that the sole function of DPNH<sub>2</sub> was to reduce FMN. It was



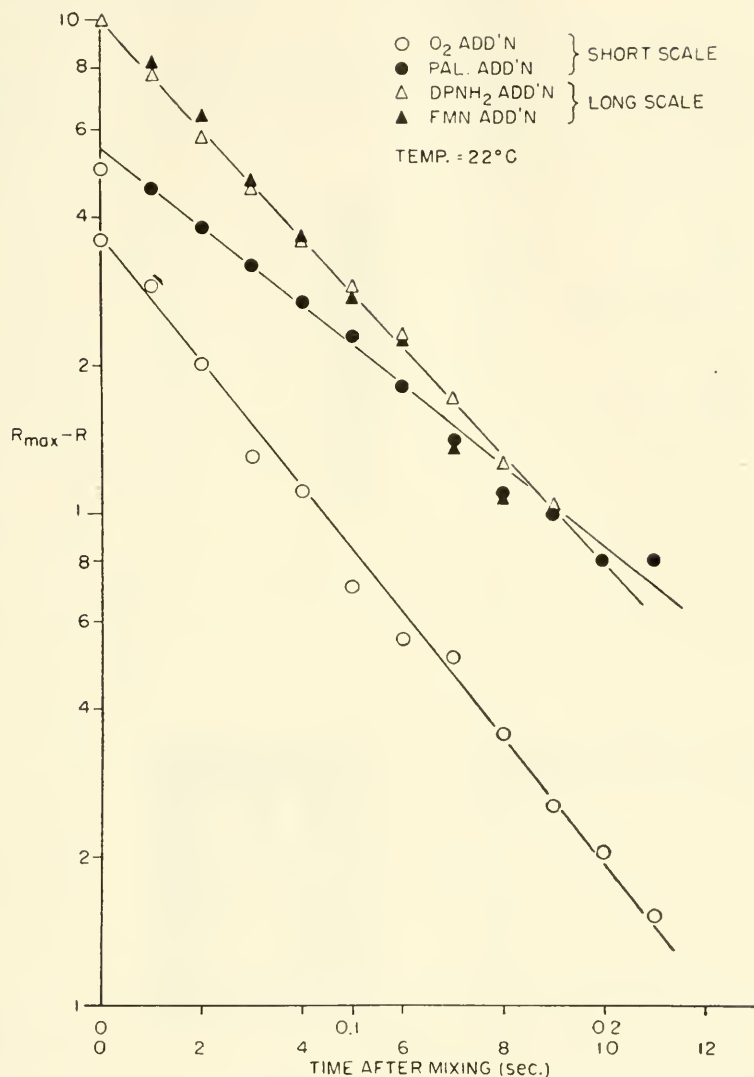


FIG. 11. Plot of data on half-rise times for oxygen, palmital, DPNH<sub>2</sub>, and FMN when each of these factors is added last to an enzyme preparation containing an excess of the other factors. The extrapolated maximum value for luminescence was taken from the records ( $R_{\max}$ ). The values for luminescence at various times after mixing the last necessary component were subtracted from  $R_{\max}$  and plotted on a logarithmic axis against the time after mixing.

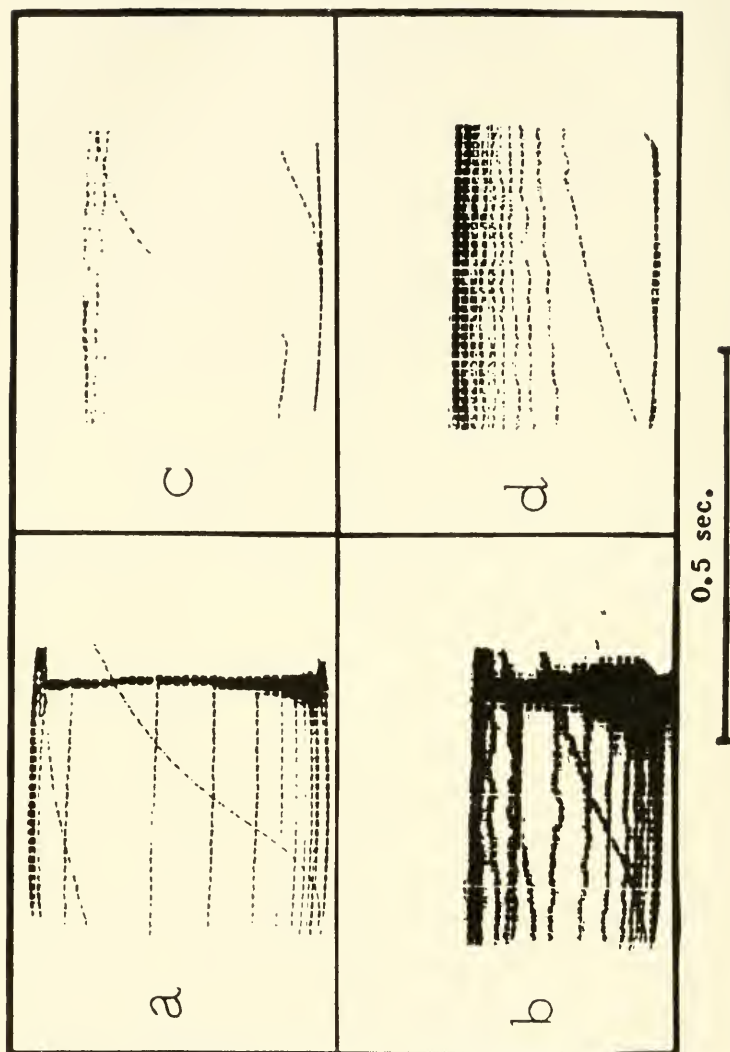


FIG. 12. Time course of luminescence when reduced riboflavin or reduced FMN were added to *A. fischeri* extracts with and without decaldehyde (KCF). The figures show that not only the total luminescence but also the time required for luminescence to approach a given percentage of the maximum value is affected by the presence of aldehyde. a, Riboflavin with KCF; b, riboflavin without KCF; c, FMN with KCF; d, FMN without KCF.

thought likely that reduced FMN should support luminescence quite as well as the oxidized flavin plus reduced DPN. This was found to be the case (Strehler *et al.*, 1954). Moreover, it was noted that maximal luminescence in the presence of reduced flavins and rapid rises to the high level of luminescence as well as rapid utilization of the reduced flavins occurred only in the presence of aldehyde. Thus, the aldehyde functions subsequently to FMN reduction (see Fig. 12).

In these studies we found that reduced riboflavin as well as reduced FMN will support luminescence, and we have recently reported this to be true. However, further studies by Dr. Harvey (personal communication) and Dr. McElroy (personal communication) independently have shown that the effect of adding reduced riboflavin to the system may be an indirect one although the time required for the reaction is relatively short and of the same order of magnitude as the time required for luminescence to become half-maximal when reduced FMN is added. Perhaps the reduced riboflavin reacts with the reduced FMN or possibly the reduced riboflavin can react equally well as FMNH<sub>2</sub> in one of the steps. However, reduced FMN may be required for another step either because of enzymatic specificity or because the phosphate group may be required for the formation of an active chemically conjugated intermediate as McElroy has suggested (personal communication). Harvey has also tested a number of other compounds closely related to reduced FMN such as reduced FAD, lumichrome, reduced lumiflavin, and several nonmetabolic flavins. As indicated in comment, he found that flavin mononucleotide was required for the luminescent reaction to occur at high rates.

### Pressure Effects

A large amount of effort has been expended over a number of years on the effects of pressure on the luminescence of intact luminous bacteria by Johnson, Eyring, and their collaborators (1942, 1945, 1947, 1954). Dr. Johnson will discuss in more detail some of the observations I am going to mention. However, inasmuch as they are consistent with and suggest an interpretation for some of the other observations mentioned above, it is fitting that they should be presented briefly. The main results in idealized form are abstracted in Fig. 13. When pressure is applied either to intact bacteria or to lumi-

nous extracts obtained therefrom, there is an immediate or nearly immediate increase in the level of luminescence to some higher level, which then slowly decays over a period of seconds or minutes to some lower steady state level. When the pressure is released, the exact converse effects occur, i.e., the luminescence drops nearly instantaneously to a lower level and then slowly rises to the level of luminescence obtaining prior to the application of pressure. The most striking modifier of this course of events is the long-chain aldehyde. In the absence of long-chain aldehyde the luminescence is virtually unaffected at suboptimal temperatures by the application or release of pressure. The height of the spike as well as the steady state level under pressure are dependent on aldehyde concentration but not to a marked extent except when aldehyde is completely absent.

The exact time course of the luminescence also depends on the concentration of KCF. Suboptimal amounts of this component produce effects which are roughly similar to the course of luminescence in the presence of saturating amounts of the aldehyde, but there are some rather notable differences. Initially, the level of luminescence rises abruptly, as is the case in the system saturated with aldehyde, but it almost immediately decays to a value near to the starting point and then slowly and probably exponentially decreases to a lower level.

In order to identify the step or steps which react in this manner when pressure is applied, the following experiments were performed. First, in order to determine whether the luminescence "spike" which occurs when pressure is applied is due to the latter steps in the sequence or to earlier ones, we introduced reduced flavins into the pressure chamber, and upon applying and releasing pressure it was noted that the luminescence rose when pressure was applied but no marked decrease to a new steady state was evident above the normal decay of the luminescence when flavin was added (i.e., the flavin disappears rather rapidly since it is consumed both by enzymatic reactions and by nonenzymatic auto-oxidations). Secondly, when the pressure was released, the luminescence dropped almost immediately to a new lower level and did not rise subsequently. It was thought that the slow decay and rise might be identified with one of the time constants noted in the rise time experiments discussed earlier, and it was possible to show that the time required for the luminescence to

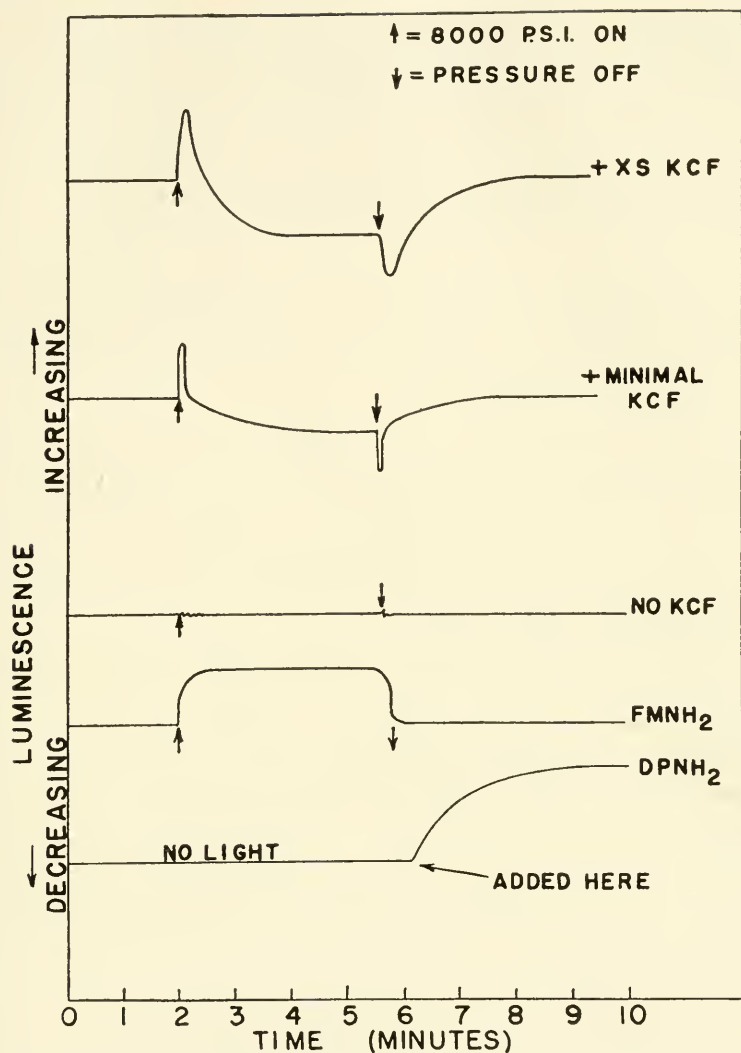


FIG. 13. Idealized representation of the effects of hydrostatic pressure on bacterial extract luminescence. Reading from the top down, the figure illustrates the effect of pressure application and release in the presence of excess decaldehyde (ca.  $10 \mu\text{g/ml}$ ); in the presence of limiting concentrations of aldehyde (ca.  $0.37 \mu\text{g/ml}$ ); in the absence of added aldehyde; when  $\text{FMNH}_2$  is the substrate (aldehyde present); and finally (for comparison with the top curve), the time course of luminescence when  $\text{DPNH}_2$  (or FMN) is added last. Temperature ca.  $16^\circ \text{C}$ .

reach one-half of the steady-state value when pressure was released was similar to if not identical with the time required for the luminescence to reach one-half its maximum rate when reduced DPN was the last component added. Thus it seems clear that the reaction which is inhibited by pressure is the  $\text{DPNH}_2$ -FMN reaction and that flavin oxidation via the luminescent pathway is potentiated by pressure. The gradual decrease in luminescence after the initial spike when pressure is applied, can be viewed as the decrease in the reduced flavin mononucleotide pool size and the converse effect, the slow rise following the "black out," is apparently due to the rise in the size of this same pool as a consequence of the greater rate of the  $\text{DPNH}_2$ -flavin reaction when the pressure is released.

The simplest interpretation of the observed effects is that (1) the reduction of flavin by reduced DPN proceeds with a volume increase on activation and that the application of high hydrostatic pressure retards this process, while (2) the oxidation of flavin in the luminescent reaction proceeds with a volume decrease on activation and is thus accelerated under pressure.

It should be pointed out that these are not the exclusive possible interpretations of the observed effects. Although the effect of pressure on the luminous oxidation of flavin is nearly instantaneous, at low temperatures it is clear that there is considerable delay in reaching the small spike maximum. If the effect were on the final reaction step, it would seem unlikely that such a period of time should be required for the rate of the reaction to become maximal. Moreover, a net increase in the reaction rate under pressure does not necessarily mean that the activation step is pressure sensitive. Another possibility is that some intermediate of flavin oxidation dissociates from an enzyme with a volume increase and further gives rise to a nonluminous reaction. Under these conditions the application of pressure would prevent the intermediate from being dissociated from the enzyme, raise its concentration and result in the increased luminescence observed. The same type of argument may be applied to the observed effect on the  $\text{DPNH}_2$ -FMN reaction in which it might be argued that the association constants of one of the reactants might be appreciably modified by pressure. If this is the meaning of the observation, the association of the substrate with the enzyme would involve a net vol-



ume increase. The observations just noted will be included in the following section on the mechanism of bacterial luminescence where an attempt will be made to present a coherent picture of all the facts thus far presented.

## Mechanism of Bacterial Extract Luminescence

### Physical Considerations

Before proceeding with a proposed specific chemical mechanism derived from the above experiments and inferences therefrom, it is important to state a number of restrictions imposed on any model mechanism of luminescence by physical considerations of various types.

First of all there is the restriction imposed by the thermodynamic aspects of the reaction. The light emitted corresponds to between 52 and 60 kcal at the maximum energy region of the spectrum. Thus it would seem unlikely that any reaction furnishing less than around 50 kcal of energy would be capable of producing luminescence in the extracts. Second, the rate of the reaction must also be sufficient to account for the observed yields of 1/100 to 1/1000 light quanta produced per oxygen consumed and the real activation energy of any given step cannot be so high that the observed rates are prohibited (Cormier and Strehler, 1954). If the apparent activation energies are higher than would permit such a reaction to proceed at a reasonable rate, it must be concluded that some artifactual influence produces the extremely high observed activation energies.

Although it is theoretically possible for a reaction liberating only around 50 kcal to emit light of somewhat more energetic character, this is a rather anomalous occurrence, and it is the instinct and experience of physical chemists to expect an even larger release of energy by a considerable number of kilocalories to be prerequisite to the occurrence of a chemiluminescent reaction.

### Discussion and Conclusions

With these preliminary thermodynamic and kinetic considerations in mind, we would like to present the scheme illustrated in Fig. 14 which furnishes a framework for interpreting the observations which

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aldehyde nec.

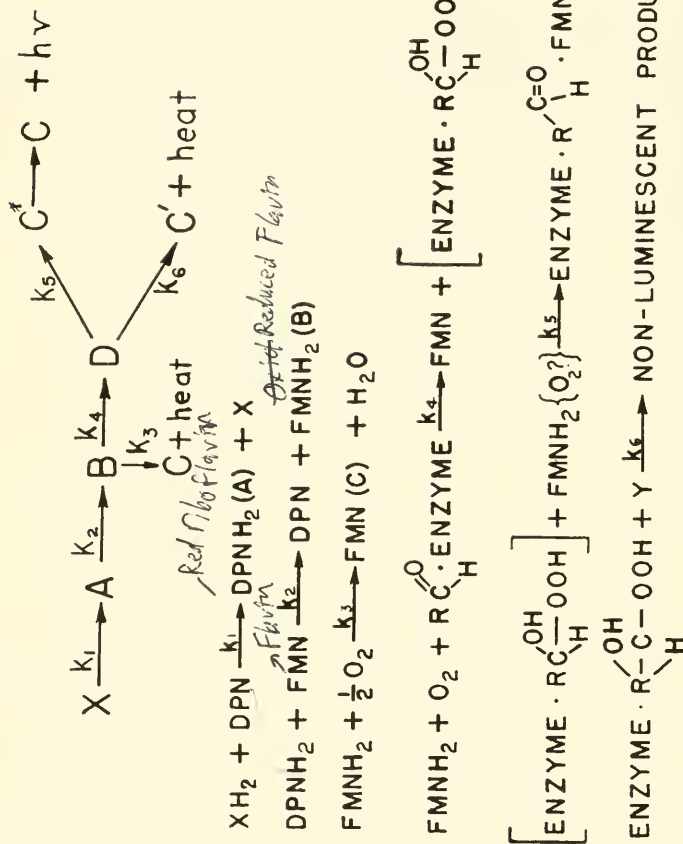


Fig. 14. A proposed detailed scheme of reactions leading to bacterial extract luminescence, attempting to account

for the major observations discussed in the text.  $RC(=O) \cdot OOH$  represents an aldehyde-peroxide addition prod-

uct analogous to the aldehyde hydrate. The  $O_2$  in braces indicates that the requirements for oxygen in this reaction are unknown. The products of  $k_3$  include, of course, 2 water molecules. Component D is a hypothetical intermediate, and in this scheme the aldehyde is regenerated if luminescence occurs.

we and others have made on *in vitro* luminescence. Since it is possible to obtain luminescence with reduced flavins as substrate and since the oxidation of this compound by molecular oxygen liberates only 37 kcal (approx.) per mole of flavin oxidized (to water and oxidized flavin), it seems unlikely that the oxidation of a single flavin molecule would furnish enough energy to produce luminescence and indeed this fact may be responsible for the fact that most flavin auto-oxidase catalyzed reactions do not proceed with luminescence.

Thus it seems highly probable that the energy released when more than one flavin molecule is oxidized must be channeled into a single flavin molecule (Strehler and Shoup, 1953; McElroy and Strehler, 1954). How could this be accomplished? According to work of Drew, the luminescent reaction involving peroxide and 3-aminophthalhydrazide is in fact a dismutation reaction between two peroxide molecules with the fluorescent-chemiluminescent molecule acting only as an intermediary in the process (Drew, 1939). Similarly, riboflavin chemiluminesces in the presence of peroxide and a metallic activator. Inasmuch as the chemiluminescent emission of this reaction is quite similar to the fluorescent emission of oxidized flavin, it seems rather unlikely that the main chromophoric grouping of the flavin molecule is destroyed during the reaction. Rather, flavin may be acting as a catalyst for peroxide decomposition.

In bacterial luminescence we would postulate that the reduced flavin formed by reaction between  $\text{DPNH}_2$  and oxidized flavin is oxidized with the intermediate formation of a compound equivalent to but perhaps not identical with peroxide and that this peroxide either reacts with another peroxide in intimate association with the flavin molecule and constituent protein of the luciferase, or that the peroxide analog thus formed oxidizes another reduced flavin molecule. The dismutation of two peroxide molecules to form oxygen and water liberates about 50 to 54 kcal, which is approximately the energy required to excite a molecule to emit in the blue-green region of the spectrum. The scheme presented in Fig. 14 represents the most consistent interpretation of the data we have been able to formulate. In this scheme DPN is reduced by some metabolic intermediate such as malic acid or typical Embden-Meyerhof components. Reduced DPN thereupon reacts with oxidized FMN in the presence of a diaphorase

type enzyme and the flavin then is oxidized in the luminescent flavin oxidase. According to this picture aldehyde functions in this sequence by forming an oxygen adduct (Jockusch, 1949; McDowell and Thomas, 1949; Wittig and Pieper, 1941), which oxidizes the flavin forming in essence a peroxide aldehyde addition product.\* This peroxy aldehyde then oxidizes another flavin or, as mentioned alternatively, peroxide with the subsequent emission of light.

The slowest half-rise times are elicited by the DPN-substrate reactions; the intermediate rise times occur when the  $\text{DPNH}_2$ -FMN reaction is the starting point; and the more rapid rise times are characteristic of the main luminescent reactions involving a somewhat complicate mechanism of  $\text{FMNH}_2$  oxidation.

The pressure studies give some clue as to the site of action of the aldehyde. Among the observations which must be fit into any scheme which purports to present a unified picture are included the fact that reduced flavin requires aldehyde for maximal luminous oxidation, that the pressure effects are not observable in the absence of aldehyde, and that limiting amounts of aldehyde produce an anomalous type of pressure response. Since reduced flavin is easily auto-oxidizable by oxygen the lack of effect of KCF on the rate of respiration at high oxygen tensions is simply due to the fact that auto-oxidation of the flavins maintains the respiratory rate.

When aldehyde is added, however, the pathway is considerably different. The flavin is oxidized by the aldehyde oxygen addition product, and the pool size of reduced FMN is diminished. The intermediate "D" represents the hypothetical aldehyde peroxide addition product, which is formed only in the presence of KCF and reduced flavin. If the concentration of aldehyde is nil or vanishingly small the amount of D which accumulates is minuscule and, therefore, since the reaction B to D is limiting, the rate of light output cannot be accelerated by pressure. Moreover, since the pool size of  $\text{FMNH}_2$  will remain large, any B to D enzyme remaining will be essentially

\* We have recently synthesized in our laboratory the crystalline peroxide-nonanaldehyde addition product ( $\alpha$ -oxynonylhydroperoxide) and find its luminescence potentiating effect to be several times that of the free aldehyde on a molar basis. Although this observation does not necessarily support the scheme as set forth, it is interesting that this derivative is more effective in promoting luminescence than the free aldehyde.

saturated with respect to  $\text{FMNH}_2$ . At intermediate concentrations of aldehyde, the concentration of  $\text{FMNH}_2$  drops considerably, but the amount of D which accumulates is relatively small and the slowest step is B to D. When pressure is applied therefore, there is an instantaneous small increase in luminescence which soon decays to its original rate, under which conditions the rate of D to C is again determined by B to D. However, since pressure affects the amount of reaction A to B ( $k_1$ ) the pool size of B soon drops and the reaction B to D slows as does D to C. This proposed scheme is consistent with the fact that the presence of aldehyde seems to be required for maximal respiration and luminescence at low oxygen tension, which effect can be viewed as a result of  $\text{O}_2$  binding by the aldehyde. Finally, this scheme furnishes a plausible mechanism for the conservation of energy in intermediates preparatory to the final light-emitting step.

One of the weaknesses of the scheme which has been presented revolves about the energetic aspects of the process. Although peroxide dismutation to water and oxygen liberates about 50–55 kcal and the oxidation of reduced flavin by peroxide would presumably yield (37 to 40 plus 25) or 62 to 65 kcal, neither of these processes by itself would seem to be exergonic enough to support luminescence of a maximum energy per einstein roughly equivalent to 62 kcal. This cautious view is based on the fact that chemiluminescent reactions in general would be expected to require for their occurrence a considerable excess of energy over the energy stored temporarily in the excited state, since no energy transfer process is likely to proceed without incidental losses.

On the other hand, it is possible that the net free energy change in the chemical reaction prerequisite to an excited molecule need not represent the total energy available for the formation of the excited state. Conceivably thermal energy could also contribute substantially to the total energy budget in at least two ways. Since experimentally it is known that the luminescent reaction proceeds with an appreciable activation energy, all or a part of this energy may be available and be added to the energy supplied concurrently by the net energy release during reaction. Thus, if the activation process for oxidation by the luminescent path involves an atomic configuration in which the elec-



trons are already displaced toward the excited level, this thermally derived energy would be available for the formation of the excited state, in addition to the energy liberated on reaction.

Another possible mechanism involves the formation of an unstable oxidizing free radical (e.g., enzyme·RCHO·HO<sub>2</sub>·) at the expense of thermal energy. Formation of such a compound might represent a major portion of the observed activation energy and, upon reaction with a flavin, give rise to a much more exergonic step reaction than would be indicated by the overall nonluminous process. The energy thus derived would in all likelihood be sufficient to excite the fluorescent molecule.

Unfortunately, evidence is not at present available to assay critically the merits, faults, or pertinence of the various possibilities discussed. The biochemical evidence can, in a large part, be molded into a consistent picture. Whether the physical objections to the biochemical deductions are fatal, must be resolved by further experiment.

#### ACKNOWLEDGMENTS

The author wishes to point out that a large portion of this work would not have been carried out so promptly and efficiently were it not for the able collaboration of Mr. Milton Cormier of the Oak Ridge National Laboratory Biology Division. Dr. John R. Totter and Dr. William A. Arnold are to be thanked for invaluable assistance and advice during the course of the work. The latter and Dr. James Franck, of the University of Chicago, contributed liberally of time and advice on certain points in the manuscript as did Dr. Frank Johnson, of Princeton University, although the ideas expressed do not necessarily coincide with theirs.

Much of the work with bacterial extracts on which this review is based was performed at the Biology Division of the Oak Ridge National Laboratory and in collaboration with Drs. E. Newton Harvey, Joseph Chang, and Frank Johnson of Princeton.

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## DISCUSSION

### Bacterial Luciferin

**Dr. Harvey:** The striking luminescence which appears when reduced flavin mononucleotide (reduced riboflavin phosphate,  $\text{FMN} \cdot \text{H}_2$ ) is added to cell-free luminous bacterial extracts containing a long-chain aliphatic aldehyde is easily visible to the dark adapted eye and reminds one of the luciferin-luciferase reaction of other organisms. The experiment raises a number of questions: (1) whether the  $\text{FMN} \cdot \text{H}_2$  is reducing some other compound in the bacterial extract which is responsible for the light; (2) whether other reduced flavin compounds will luminesce when mixed with bacterial extracts; (3) whether  $\text{FMN} \cdot \text{H}_2$  may be regarded as bacterial luciferin. The first question has been answered in the negative by showing that a reduced compound with a lower redox potential than  $\text{FMN} \cdot \text{H}_2$  ( $E'_0$  at pH 7 =  $-0.185$ ), when added to the bacterial extract, does not cause light emission. Strehler *et al.* (1954) found that reduced anthraquinone-2-6-disodium sulfonate ( $E'_0$  at pH 7 =  $-0.192$ ), a substance harmless for luminous bacteria (Harvey, 1929), evokes no luminescence when mixed with the bacterial extract. However, if oxidized riboflavin phosphate (FMN) has been previously added, then the reduced anthraquinone reduces the FMN and a bright light appears. The experiments suggest that reduced FMN may be designated bacterial luciferin.

In the same paper it was reported that riboflavin (Merck) emitted light when mixed with cell-free bacterial extracts of *Achromobacter fischeri*. Recently I have tested a number of other flavin compounds with a crude cell-free extract of a luminous bacterium having a low ( $12^\circ \text{C}$ ) temperature optimum. The cell-free extract was prepared by Dr. B. L. Strehler from a strain of luminous bacteria obtained from Dr. C. B. Van Niel and known as "Gest." The active acetone bacterial powder had been extracted with water and centrifuged at  $70,000 \times g$  for one hour. Although the temperature rose somewhat during centrifuging, the water extract always gave a bright light when mixed with reduced riboflavin phosphate, indicating that the enzyme essential for light production had not been denatured. The extract was stored at  $-10^\circ \text{C}$  before use. The dilute flavin solutions in distilled water were reduced in small centrifuge tubes in a stream of hydrogen after adding platinized asbestos. The tubes were then stoppered, centrifuged at

1200  $\times$  g for 5 minutes to throw down the asbestos, and the reduced flavin supernatant removed with a 1-cc tuberculin syringe and 0.2 cc squirted under 0.6 cc of the bacterial extract containing a small amount of palmitaldehyde. This method is simple and has many advantages for handling solutions of rapidly oxidizable substances. Observation of fluorescence in ultraviolet light indicates that the flavin oxidizes only at the surface of such a solution in contact with air and not in the syringe.

TABLE IV

Reduced flavin	Source	Luminescence with centrifuged cell-free bacterial extract (Gest) plus palmitaldehyde
Riboflavin phosphate (FMN)	Sigma Chem. Co.	Bright
Flavin adenine dinucleotide (FAD)	Sigma 15	None
Moccasin snake venom, a source of L-amino-acid oxidase	H. R. Mahler	None
DPNH cytochrome c reductase (liver)	H. R. Mahler	None
Lumichrome (dimethylalloxazine)	Merck & Company	Very faint
Lumiflavin (trimethylisalloxazine)	Bios Labs.	None
L-araboflavin	L. Michaelis	None
Glucisoalloxazine	L. Michaelis	None
N-methylalloxazine	L. Michaelis	Fair
Riboflavin	Merck & Co.	Faint
Riboflavin	Gen. Biochem. Inc.	None

The results of adding various flavins to the crude Gest extract are shown in Table IV. The FMN experiment served as a control to indicate that the extract was active. It will be observed that with the exception of riboflavin phosphate and N-methylalloxazine, practically no luminescence appears with any flavin. Samples of riboflavin from two different sources gave different results. To what extent contaminants may be responsible for luminescence with riboflavin and with lumichrome is impossible to say. The negative results with the flavoproteins, both of which contain FAD (flavinadeninedinucleotide) or an FAD like prosthetic group, should be especially noted. These flavoproteins were lyophilized and kindly supplied by Dr. H. R. Mahler, of the University of Wisconsin Institute of Enzyme Research. It will be most important to test a flavoprotein containing FMN.

Regarding the question as to which substance among a number necessary for luminescence is to be regarded as luciferin, I would like to quote from a paper by Harvey and Tsuji, entitled "Luminescence of *Cypridina* luciferin without luciferase, together with an appraisal of the term, luciferin," now in press in the *Journal of Cellular and Comparative Physiology*:

Rather than placing the emphasis on a limiting factor, or on heat stability or dialyzability or even oxidizability [as an indication of luciferin], as has been done previously, light emission should be the criterion. In the case of luminous organisms requiring dissolved molecular oxygen for luminescence, luciferin may properly be defined as the oxidizable substance supplying molecules capable of absorbing enough excess energy to emit in the visible region. Such a definition implies that some form of luciferin molecule—either free base or acid, either dissociated anion or cation, in reduced or oxidized form, either free or combined with protein, like a prosthetic enzyme group—can pick up the energy of the oxidative reaction in which it is involved. Such a definition does not mean that luciferin is the same substance in different luminous animals, nor does it necessarily designate luciferin molecules themselves as the ones which emit, but it does imply that a related molecule, such as a luciferin-luciferase combination, or an oxidized luciferin molecule, or a molecule of an intermediate step, is the emitter. The molecule actually emitting might be referred to as the photogen. . . . It has long been recognized that a substance, whose molecules are readily excited to fluoresce by the energy of radiation, is most likely to be chemiluminescent from the energy of a chemical reaction. . . . Therefore it seems most logical to regard the reduced form of the fluorescent flavin as bacterial luciferin rather than the non-fluorescent aldehyde. Although riboflavin and FMN are not fluorescent in the reduced form, the oxidized flavins fluoresce yellow green over a wide pH range, from pH 1 to 11. An analogous situation is to be observed among the ctenophores, where a striking phenomenon is the fluorescence of the luminous organ after, but not before bioluminescence has occurred, as if the final product of luminescence was a fluorescent molecule (Harvey, 1925).

Reduced FMN is comparable to *Cypridina* luciferin in that it undergoes spontaneous oxidation by dissolved oxygen without luminescence and only emits light in the presence of what may be called bacterial luciferase.

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**Dr. Johnson:** The role of peroxide in luminescent reactions is an interesting problem. A theory is given in the book (Johnson, Eyring, and Polissar, 1954) that would account for the luminescence of luminol, with destruction of  $\text{H}_2\text{O}_2$  but without destruction of the luminol, the peroxide acting to induce a quinone type of electronic structure which then radiates on returning to the freely resonating structure of the substituted or unsubstituted ring. In arriving at this theory, however, it was necessary to assume that four molecules of  $\text{H}_2\text{O}_2$  are decomposed, in the reactions leading to light emission, in order to account for the energy of the emitted light. In the scheme of reactions presented by Dr. Strehler, the decomposition of only two molecules of  $\text{H}_2\text{O}_2$  is assumed, and the free energy made available thereby is considerably short of the free energy needed for the observed light emission. To this extent, the hypothesis, as it stands, does not appear to be thermodynamically sound. The activation energy for the change between normal and activated states cannot be added to the free energy difference between the initial and final states to increase by much the available free energy of reaction.

**Dr. Eyring:** One restriction that thermodynamics places on any mechanism is that one cannot get more free energy in the form of quanta than is used up in the reactions. Thus,

$$n_1 \Delta F \geq n_2 h\nu \quad (1)$$

Here  $n_1$  is the total number of molecules reacting and  $\Delta F$  is the free energy used up per individual process;  $n_2$  is the number of quanta emitted and  $h\nu$  is the free energy of a quantum. The absolute maximum efficiency is thus

$$\phi = \frac{n_2}{n_1} = \frac{\Delta F}{h\nu} \quad (2)$$

Now such an efficiency can be approached if you use the reaction to do work reversibly storing it in a battery and then use the battery in an efficient fluorescent lamp. In principle this can also be realized



in chemical reactions as indicated in Fig. 15, similar to a diagram in our book (Johnson, Eyring, and Polissar, 1954). Thus thermodynamics will be satisfied if

$$\frac{k_2}{k_1 + k_2} = \frac{n_2}{n_1} \leq \frac{\Delta F}{h\nu} \quad (3)$$

The problem is to find actual systems where  $k_2/(k_1 + k_2)$  is this large. I know of none. I would rather believe this high efficiency is obtained by processes in which the free energy of additional reactions is used

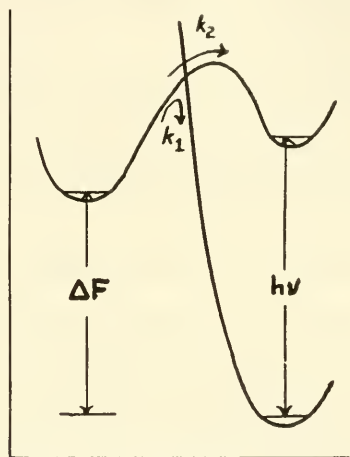


FIG. 15.

to pile up sufficient potential energy for the emission without absorbing kinetic energy from the heat reservoir by a not thermodynamically impossible but by a nevertheless unknown specialized mechanism. If one thinks of a Planck energy density versus frequency distribution curve transformed so that each abscissa, representing a frequency, is lengthened by the free energy donated by the chemical reaction to the emission process, the ordinates give the maximum intensity to be expected for the changed frequencies. The actual distribution curve can only be predicted from a detailed knowledge of reaction mechanism.

One qualitative argument against this type of efficient mechanism is to be found in the fact that kinetic energy of reaction is never passed along in an efficient manner to make thermal chains. It seems enormously more likely, as I have already stated, that enough reactions

pool their potential energy so that the kinetic energy =  $(h\nu - \Delta F)$  picked up from the heat reservoir is negligible.

You may find the papers of Audubert (1936, 1937) and of Evans, Eyring, and Kincaid (1938) interesting in this connection.

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#### On Light Energy versus Free Energy Changes in Bioluminescence

**Dr. Strehler:** In attempting to evaluate the plausibility of any overall chemical reaction as a source of the energy in a quantum emitted in a coupled luminescent reaction, the question arises: Must the  $\Delta F$  liberated per mole of the proposed reaction be greater than the energy per einstein of the emitted light? If the answer is affirmative, only those reactions furnishing more energy than that in the light need be considered as possible sources of energy for bioluminescent processes. If, on the other hand, the answer is negative, then even reactions somewhat less energetic than the light emitted cannot be ruled out as possibilities. A case in point is the oxidation of peroxide by peroxide which liberates about 54 kcal during the dismutation of 2 moles of  $\text{H}_2\text{O}_2$  and thus might be considered as a source of energy for bacterial or riboflavin chemiluminescence (max. energy/einstein = 60 kcal).

The following discussion is a qualitative series of arguments and is limited by the author's training and experience. A quantitative treatment of the thermodynamic and kinetic aspects of this problem has kindly been made by Dr. Joseph Mayer in the following portion of this discussion.

#### Premises

- (1) The chief premise is simply that thermal energy may contribute

to the energy prerequisite to the emission of a light quantum. Thus:

$$\Delta F/h\nu \geq 1 \quad \text{or} \quad \Delta F/h\nu \leq 1$$

but

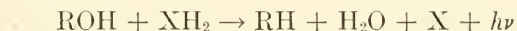
$$(\Delta F^\ddagger + \Delta F)/h\nu \geq 1$$

(2) The second premise is that partial reactions will proceed irrespective of the source of the reactants (if they are thermodynamically possible), whether the reactants are chemically or thermally generated.

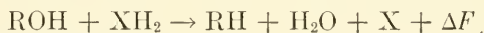
(3) The third premise is that the first premise does not violate the first or second law of thermodynamics, since not all of the energy in  $h\nu$  is available to do work.

### Mechanism

Consider a reaction:



or



in which the partial reactions are:

1.  $\text{ROH} + \text{heat} \rightarrow \text{R}\cdot + \cdot\text{OH}$
2.  $\text{R}\cdot + \text{XH}_2 \rightarrow \cdot\text{XH} + \text{RH}$
3.  $\cdot\text{OH} + \cdot\text{XH} \rightarrow \text{X} + \text{HOH} + (\text{heat or } h\nu)$

The reactions are diagrammed in Fig. 16.

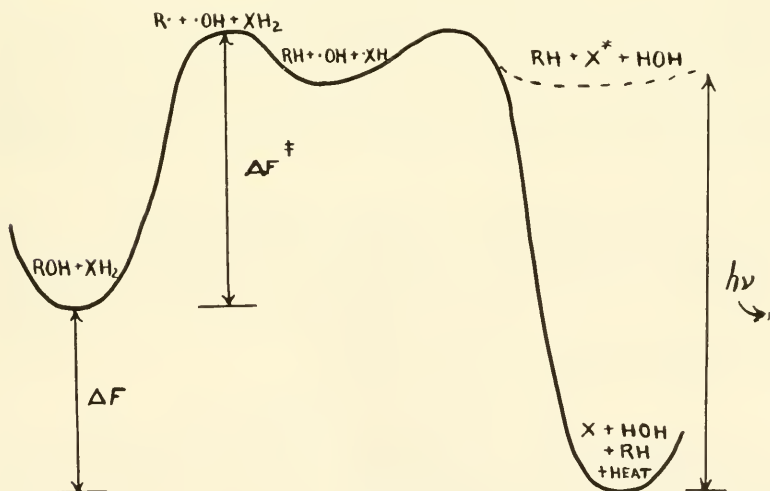


FIG. 16.

Such a reaction, if efficient, would convert thermal energy into a portion of the energy in a light quantum and  $\Delta F < h\nu$ . No objection could be raised if  $R\cdot$  and  $\cdot OH$  were generated by independent exergonic reactions and there seems no reason for assuming that reaction 3 can distinguish the source of the reactants.

### *Objections and Conclusions*

Despite the above arguments, if it could be shown that such a mechanism violated either the first or second law of thermodynamics, it would perforce be ruled out. It clearly does not violate the first law since the total energy is constant. That the proposed mechanism does not violate the second law is also clear on the following qualitative grounds and is developed quantitatively in the succeeding quantitative treatment.

If it were possible to build a 100% efficient photoelectric or photochemical device to convert the total light energy emitted by such a proposed reaction mechanism into useful work, the second law would be violated. Whether such a device could in principle be constructed is the point at issue. Suppose a photoelectric device were to be operated on light emitted as black body radiation by another body at its own temperature. Since thermal energy is here converted into light energy as an intermediate (some, though a minute amount in the wavelength region of bioluminescences!), if such a machine were possible it would itself violate the second law.

It follows from the above and following that the overall reactions possibly leading to luminescence are not restricted to those having a greater  $\Delta F$  than  $h\nu$ . One cannot rule out as participants in and energy sources for bioluminescences certain reactions less energetic than the light emitted.

### On the Maximum Efficiency of a Photochemical Reaction\*

**Dr. Mayer:** We consider the photochemical reaction:



and consider that the free energy change

$$-\Delta F^\circ = F^\circ(C) - F^\circ(B) - F^\circ(A) \quad (2)$$

\* Dr. Joseph E. Mayer, of the Institute of Nuclear Studies, University of Chicago, kindly contributed this paper, by invitation, for additional discussion on the question at issue [Ed.].

is known for the chemical reaction with the reactants and products at unit concentration.

The reaction may actually go to emit a range of frequencies,  $h\nu$ , but we suppose the mechanism for the emission of any single frequency  $\nu_0$  to be such that one molecule of A plus one of B follow some successive steps to emit one quantum,  $h\nu_0$ , uncoupled with any parallel steps by which molecules A and B react, either without the emission of light, or with emission of lower frequencies  $\nu'$ . The rate  $R_0$  of photons of frequency  $\nu_0$ , per unit frequency range, per cubic centimeter, at unit concentration of A, B, and C is now measured:

$R_0 d\nu dV$  = number of photons of frequency between  $\nu_0$  and  $\nu_0 + d\nu$  emitted by the reacting mixture of volume  $dV$ , at temperature  $T$ , with A, B, and C at unit concentration.

The question now is: What is the maximum value of  $R_0$  for any frequency,  $\nu_0$  at  $T$ , in terms of the free energy liberation,  $-\Delta F^{(0)}$ , of the chemical reaction?

The question can be answered by considering the following hypothetical reversible machine.

We place the reacting mixture, at temperature  $T$ , unit concentration of A, B, and C in a vessel and, with a negative catalyst, inhibit any reaction which emits other frequencies than  $\nu_0$ , or which does not emit light. Since the reaction which emits  $\nu_0$  is supposed to be uncoupled to any other reaction, this fiction is permissible and is equivalent to the assumption of microscopic reversibility.

We surround the reaction vessel with an insulating wall, transparent to the frequency  $\nu_0$ , but to no other radiation. The space outside of this contains black body radiation enclosed in completely reflecting walls, fitted with a completely reflecting piston, so that by compression or expansion, the temperature  $T^*$  of the radiation can be altered.

An auxiliary machine can be used to remove radiation from the reservoir at  $T^*$ , by adiabatic expansion to the temperature  $T$  of the reacting mixture it can obtain useful work, and deliver the remaining radiation to a reservoir at  $T$ . This process can be carried out reversibly, the useful work  $\Delta W$ , obtained from the radiant energy  $\Delta E^*$  taken out at  $T^*$ , is

$$\frac{\Delta W}{\Delta E^*} = (T^* - T)/T^* \quad (3)$$

By adjusting the temperature  $T^*$  of the surrounding black body radiation, we can keep the radiation density  $U(\nu_0)$  in the reaction

vessel, which will be equal to that in the black body surroundings:

$$U(\nu_0) = (8\pi h\nu_0^3/c^3)[e^{h\nu_0/kT^*} - 1]^{-1} \quad (4)$$

just such as to balance the forward and backward rate of reaction (1).

We thus have a reversible machine by which the maximum useful work obtainable from the chemical reaction (1) can be evaluated through the light emitted. We have

$$\begin{aligned} -\Delta F^{(\circ)} &= -\Delta A^\circ - \Delta PV \geq N_0 h\nu_0(T^* - T)/T^* - \Delta PV \\ &\cong N_0 h\nu_0(T^* - T)/T^* \end{aligned} \quad (5)$$

since we can assume the  $\Delta PV$  of the reaction to be negligible.

The temperature  $T^*$  of the surrounding black body radiation bath, which would just reverse reaction (1), is related to  $\nu_0$  and the rate  $R_0$  of the emission of photons in the absence of the reversing bath.

Let  $\sigma$  be the absorption cross section of molecules  $C$  for light of frequency  $\nu_0$  to reverse Eq. (1). The reverse reaction will go at a rate

$$R_r = [I(\nu_0)/h\nu_0]C\sigma N_0/V \quad (6)$$

with  $N_0$  = Avogadro's number, and  $V$  the volume (in cubic centimeters) of one mole of material at the unit concentration used in computing  $-\Delta F^{(\circ)}$  of Eq. (2). We use (4) for  $U(\nu_0)$  in (6) setting  $R_r$  equal to  $R_0$  to compute  $T^*$  as

$$h\nu_0/kT^* = \ln \left[ \frac{2}{\pi} \frac{\sigma}{\lambda_0^2} \frac{N_0}{V} \frac{1}{R_0} + 1 \right] \quad (7)$$

with

$$\lambda = c/2\pi\nu_0 \quad (8)$$

The dimensionless quantity,

$$X_0 = \sigma/\lambda_0^2 = 4\pi^2\nu_0^2\sigma/c^2 \quad (9)$$

has a maximum value of unity at resonance, but would normally be expected to be very much smaller for visible light and ordinary molecules, say of the order of  $X_0 \cong 10^{-2}$  to  $10^{-4}$ . If the concentration units of Eq. (2) are moles per liter, then  $N_0/V = 6 \times 10^{20}$ . The rates  $R_0$ , photons per cubic centimeter per second per unit frequency range may be of order  $10^6$ . The quantity  $X_0(N_0/V)/R_0$  is of order  $10^{11}$  to  $10^{13} \gg 1$ . We can neglect the unity under the logarithm of Eq. (7). Setting

$$-\Delta F^{(\circ)}/N_0 h\nu_0 \geq 1 - (T/T^*) \quad (10)$$



from (5), and

$$T - T^* = (kT/h\nu_0) \ln \frac{2}{\pi} X_0 N_0 / V R_0 \quad (11)$$

from (7),

$$\ln \frac{2}{\pi} X_0 N_0 / V R_0 \geq \frac{h\nu_0}{kT} - \frac{(-\Delta F^{(\circ)})}{N_0 kT} \quad (12)$$

we arrive at

$$R_0 \leq \frac{2}{\pi} X_0 \frac{N_0}{V} e^{-[h\nu_0 - (-\Delta F^{(\circ)})/N_0] / kT} \quad (13)$$

It appears that values of  $X_0 N_0 / V$  of  $10^{19}$  are not unreasonable, integrated over any small frequency range. Even if

$$[h\nu_0 - (-\Delta F^{(\circ)})/N_0] / kT \cong 30$$

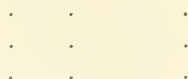
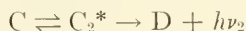
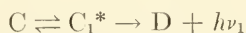
$$N_0 h\nu_0 - (-\Delta F^{(\circ)}) \cong 18 \text{ kcal, } T = 300^\circ$$

values of  $R_0$  (integrated over a short frequency range) of the order of  $10^6$  photons per cubic centimeter per second would not seem to violate the second law in any way.

Equation (13) refers to the rate  $R_0$ , at  $h\nu_0$ , of emission of photons at the concentration of reactants used in the computation of  $\Delta F^{(\circ)}$ . If the actual concentrations are lower either the rate should be corrected to unit concentration, or the  $\Delta F^{(\circ)}$  should be computed from the actual concentrations employed. In either case the equation will be the same.

### Chemiluminescence from Thermally Activated Intermediates

**Dr. Kauzmann:** Suppose that a substance can exist in two states, C and D, and that the change from state C to state D proceeds through thermal activation to any one of a series of intermediates,  $C_1^*$ ,  $C_2^*$ , . . . , each of which then produces state D by emitting a quantum of radiant energy  $h\nu_1$ ,  $h\nu_2$ , . . . , respectively, according to the following scheme:



It is convenient to denote the energy change per molecule of the overall reaction  $C \rightarrow D$  by  $h\nu_0$ . This is the energy of the quantum which would be emitted if a molecule in state C could be transformed

directly into one in state D with complete conversion of the energy difference into light. Similarly, let us indicate by  $h\nu_1^*$ ,  $h\nu_2^*$ , . . . the energy absorbed when  $C_1^*$ ,  $C_2^*$ , . . . , are formed from state C. As seen from Fig. 17, the frequencies which are actually emitted are

$$\nu_1 = \nu_0 + \nu_1^*$$

$$\nu_2 = \nu_0 + \nu_2^*, \text{ etc.}$$

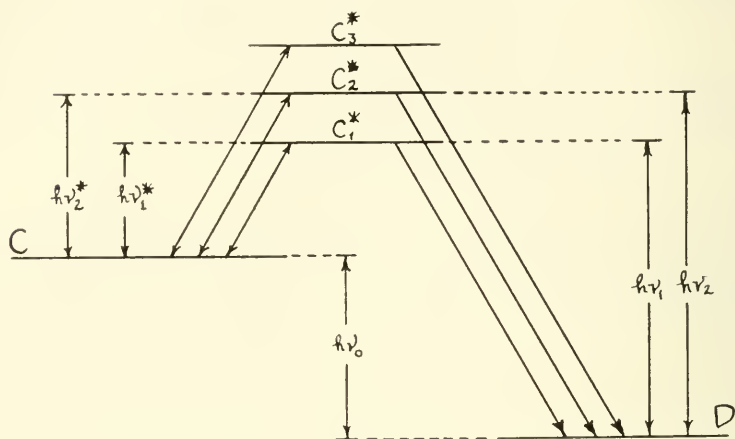


FIG. 17.

The  $\nu^*$ 's represent the contributions of the thermally activated intermediate states to the quanta that are actually emitted, while  $\nu_0$  is the contribution of the overall chemiluminescent reaction,  $C \rightarrow D + \text{light}$ .

It is obvious that there must be some thermodynamic limit to the increase in the energy of the emitted quantum above  $h\nu_0$  that is obtainable from the emission produced in this way by thermally activated intermediates. One might expect that the intensity of light emitted at a frequency  $\nu$ , greater than  $\nu_0$ , would decrease with increasing frequency in proportion to  $\exp(-h(\nu - \nu_0)/kT)$ , where  $T$  is the temperature of the system. If  $\nu_0$  corresponded to a wavelength of 5000 Å, this means that the intensity should decrease by a factor of 10 for each 120-Å decrease in the wavelength—which is a somewhat sharper edge for an emission band than is usually observed with luminous bacteria (Spruit-van der Burg, 1950). An exponential factor of this kind must indeed operate, but as we shall now show, the upper limit set by the laws of thermodynamics on the intensity is surprisingly

high, so this factor may not become important until frequencies are reached that are well above  $\nu_0$ .

Let us place one mole of the substance in state C in a vessel at temperature  $T$  whose walls are perfect reflectors of radiation, so that all the radiation emitted by the reaction is trapped inside the vessel. Eventually a steady state ought to be reached in which the rates of transitions from the states  $C^*$  to state D are balanced by absorption of quanta by molecules in state D which return them to state  $C^*$ . The density of radiation present in this steady state leads to a thermodynamically defined upper limit to the luminosity that is attainable from the conversion of C to D through the intermediates  $C^*$ .

Transitions from state  $C^*$  to state D can occur in two ways: in the first place, there is a probability,  $A_1 dt$ , that a molecule in state  $C_1^*$  will in the absence of radiation undergo a transition to state D in a small time interval  $dt$ . Secondly, if radiation is present, the probability of a transition is increased by an amount  $\rho_i B_i dt$  where  $\rho_i$  is the energy per unit volume of radiation whose frequency lies in the range between  $\nu_i$  and  $\nu_i + 1$  cycles per second. The fundamental quantum theory of electromagnetic radiation (Mott and Sneddon, 1948) shows that  $A_i/B_i = 8\pi h \nu_i^3 / c^3$ , where  $c$  is the velocity of light. Furthermore, the probability of a reverse transition,  $D \rightarrow C_i^*$ , occurring in time interval  $dt$  is equal to  $\rho_i B_i dt$ . (Since state D is assumed to have less energy than states  $C_i^*$ , there can be no transitions from  $D \rightarrow C_i^*$  in the absence of radiation.) Writing the concentrations of  $C_i^*$  and D as  $(C_i^*)$  and  $(D)$ , we see that when the steady state is reached,

$$(\rho_i B_i + A_i)(C_i^*) = \rho_i B_i (D) \quad (1)$$

Since the states  $C_i^*$  are assumed to be populated from C by thermal activation, we may make use of the equilibrium constant,

$$K_i^* = (C_i^*)/(C) = \exp(-h\nu_i^*/kT) \quad (2)$$

From (1) and (2) and the value of  $A_i/B_i$  we find for the steady state radiation density

$$\rho_i = \frac{a \nu_i^3}{\frac{(D)}{(C)} e^{h\nu_i^*/kT} - 1} \quad (3)$$

where  $a = 8\pi h/c^3$ . Note that no steady state is possible if  $(D)/(C) \leq \exp(-h\nu_i/kT)$ . Let us compare this result with the ordinary thermal

radiation ("black body radiation"). Planck showed that this is

$$\rho_i = \frac{a\nu_i^3}{e^{h\nu_i/kT} - 1} \quad (4)$$

If C and D are in thermodynamic equilibrium,  $(D)/(C) = \exp(h\nu_0/kT)$  and Eq. (3) becomes identical with Planck's formula—as of course it must. (Indeed, this is a simple and often used way of deriving Planck's formula.) If C and D are present in comparable concentrations, as they must be during most of the course of a chemiluminescent reaction, we can write  $(C) \cong (D)$ , giving

$$\rho_i \approx \frac{a\nu_i^3}{e^{h\nu_i^*/kT} - 1}$$

We see that the radiation density thus obtained is related to the black body radiation density  $\rho_i^*$  associated with the quantum  $h\nu_i^*$  in the following way:

$$\rho_i \approx (\nu_i/\nu_i^*)^3 \rho_i^*$$

That is, during a chemiluminescent reaction involving an intermediate which is activated thermally by an amount equivalent to a quantum of energy  $h\nu_i^*$ , the radiation density may be greater than the ordinary black body radiation of photons of energy  $h\nu_i^*$  at the same temperature by a factor  $[(\nu_0 + \nu_i^*)/\nu_i^*]^3$ . This factor might be quite large; for instance, if  $\nu_0$  corresponds to a wavelength of 5300 Å or an energy of 54,000 cal/mole, and  $\nu_i^*$  corresponds to an energy of 6000 cal/mole (or about  $10kT$ ), the steady state chemiluminescent radiation density at 4750 Å (corresponding to an energy of 60,000 cal/mole) would be 1000 times greater than the black body radiation at 47,500 Å (corresponding to 6000 cal/mole). The physical reason for this somewhat surprising result is that the number of states available to a photon is proportional to the square of its frequency, while its energy is proportional to its frequency. By attaching the energy  $h\nu_i^*$  to a more energetic photon of energy  $h\nu_0$  we increase the probability of finding the energy  $h\nu_i^*$  by a factor of  $[(\nu_0 + \nu_i^*)/\nu_i^*]^3$ .

In order to find the numerical value of the limiting intensity of emission, let us assume that the states  $C_i^*$  form a continuum above a threshold frequency  $\nu_1^* = \nu^*$ . Let us also assume that there is a small hole in the side of the vessel containing the chemiluminescent reaction, through which radiation may leak out. The rate of emission of energy associated with a frequency in the range from  $\nu_i$  to  $\nu_i + d\nu_i$  from such a hole is simply  $(c/2)\rho_i d\nu_i$  per unit area of the hole per unit time. For

the total rate of emission of all frequencies we find

$$\text{Total rate} = (c/2) \int_{\nu_0 + \nu^*}^{\infty} \rho_i d\nu_i = \frac{ca}{2} \int_{\nu^*}^{\infty} \frac{(\nu_0 + \nu_i^*)^3 d\nu_i^*}{\frac{(D)}{(C)} e^{h\nu_i^*/kT} - 1}$$

If  $h\nu^*$  is considerably larger than  $kT$  but still small compared with  $\nu_0$  and if  $(C) \cong (D)$ , we can obtain the approximate result,

$$\begin{aligned} \text{Total rate} &\cong \frac{c}{2} a \frac{kT}{h} \nu_0^3 e^{-h\nu^*/kT} \\ &= 5.7 \times 10^{-45} \nu_0^3 e^{-h\nu^*/kT} \text{ watt/cm}^2 \end{aligned}$$

where  $\nu_0$  is expressed in  $\text{sec.}^{-1}$ .


Let us take  $\nu_0 = 0.57 \times 10^{15} \text{ sec.}^{-1}$  (equivalent to a wavelength of 5300 Å) and  $h\nu^* = 10kT$ . At  $300^\circ \text{ K}$  this corresponds to emission at a wavelength less than 4800 Å, which is 500 Å beyond the expected threshold. We find a possible rate of emission of about  $5 \times 10^{-5} \text{ watt/cm}^2$ , equivalent to about  $10^{14}$  quanta per second per square centimeter. This is to be compared with the normal emission by a black body of radiation having quanta whose energy is more than  $10kT$ , which comes to only  $5.8 \times 10^{-8} \text{ watt/cm}^2$  at  $300^\circ \text{ K}$ . It corresponds to a relatively bright light, though not very much brighter than the observed maximum brightness of a concentrated suspension of luminous bacteria or other bioluminescent organisms, being of the order of the brightness of a white surface one meter distant from a 40-watt tungsten lamp. The total possible rate of emission beyond 4690 Å, however, is  $5 \times 10^{-6} \text{ watt/cm}^2$ , and that beyond 4600 Å is  $5 \times 10^{-7} \text{ watt/cm}^2$ . The luminescence of luminous bacteria does not drop off this rapidly with decreasing wavelength, and it would be interesting to see if the actual emission at short wavelengths surpassed the theoretical upper limit.

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## Purification and Properties of Bacterial Luciferase

J. W. HASTINGS and W. D. McELROY

Department of Biological Sciences, Northwestern University, Evanston,  
Illinois, and McCollum-Pratt Institute and Department of Biology,  
The Johns Hopkins University, Baltimore, Maryland

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The demonstration of luminescence in cell-free bacterial extracts, which had long been sought by workers in the field, was finally achieved by Strehler early in 1953. After the demonstration that diphosphopyridine nucleotide (DPN) markedly stimulated luminescence, work progressed rapidly. Within the year this bioluminescent reaction was the first in which all known stimulatory factors had been chemically identified. Although many unanswered questions concerning the mechanism of the reaction remain, it seems worth while to review some of our studies on the system, particularly where our results differ from or amplify those reported by Strehler.

Preliminary experiments with relatively crude extracts had indicated that (1) DPN was functional in the luminescent reaction only when reduced and might be replaced by other reducing compounds (TPNH), and (2) that a heat stabile fraction of the crude extract contained a stimulatory factor not identical with DPNH. The purification of the enzyme and a study of other factors was thus undertaken. Conventional enzymological methods resulted in a forty- to sixty-fold purification of the enzyme (see Table I).<sup>\*</sup> Bacteria

<sup>\*</sup> Repeated  $(\text{NH}_4)_2\text{SO}_4$  fractionations of the active fractions have resulted in even greater purity. Green and McElroy (unpublished) find that their best fractions are homogeneous by ultracentrifugation (mol. wt. ca. 85,000) and contain three components with slightly different electrophoretic mobilities at pH 7.6. The active fraction constitutes over 70% of the total protein.

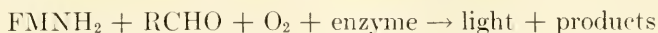
TABLE I  
Purification of Bacterial Luciferase

Fraction	Protein/ml, mg	Light Units/ml	Specific Activity, Light units/mg protein	Recovery, %
Crude	1.2	17.5	14.1	—
Acid precipitate	11.2	193.	17.3	110
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fractions				
40-50%	0.67	0.85	1.27	0.3
50-60%	1.36	26.	19.	10.0
60-70%	1.58	244.	155.	94.5
70-80%	0.50	15.5	31.	6.0

(*Achromobacter fischeri*) grown on Farghaly's (1950) liquid medium with 1% peptone added were harvested by centrifugation after 12-18 hours and lysed in distilled water (3 g wet weight per 100 ml H<sub>2</sub>O). After removal of the cell debris by centrifugation the protein was precipitated by acidification and resuspended in a small volume of water at pH 7.0. Further purification was achieved by fractionation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 7.0. All procedures were carried out in the cold. Light intensity was measured with a photomultiplier apparatus (Hastings, McElroy, and Coulombre, 1953). The reaction mixture consisted of 0.5 ml of buffer (0.135 M KH<sub>2</sub>PO<sub>4</sub>, 0.135 M Na<sub>2</sub>HPO<sub>4</sub>, and 0.01 M NaCl) pH 6.8, 0.1 ml of  $2.5 \times 10^{-4}$  M DPNH, 0.1 ml of  $5 \times 10^{-5}$  M riboflavin-5-phosphate, 1.0 ml of a saturated water solution of dodecyl aldehyde, and enzyme, other reagents, and water to a total volume of 2.5 ml. The reaction was usually initiated by adding DPNH.

The enzyme preparation from the 60-70% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction is colorless, rapidly destroyed at 80° C, and stable for several months in the frozen state. It will emit a weak light with DPNH alone, but for appreciable activity FMN (McElroy, Hastings, Sonnenfeld, and Coulombre, 1953) and aldehyde (Cormier and Strehler, 1953) must be added. FMN reduced by bubbling hydrogen in the presence of a catalyst will dispense with the requirement for DPNH (Strehler, Harvey, Chang, and Cormier, 1954). Other reduced compounds (reduced riboflavin, reduced dyes) will function in lieu of DPNH, but FMN is

a specific requirement (McElroy and Green, unpublished). The purified enzyme acts as a typical diaphorase (McElroy and Green, 1954). The overall luminescent reaction may be written:



None of the products has been identified as yet, although we have evidence that the aldehyde is destroyed during the reaction. The pH optimum for this reaction is 6.9 (Fig. 1). The luminescent reaction

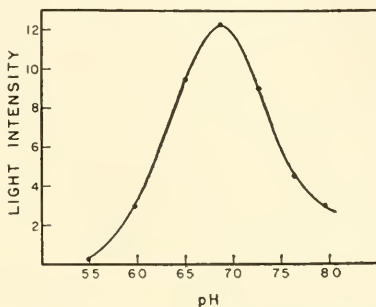


FIG. 1. Effect of pH upon bacterial luminescence. Buffer as described in text.

does not involve free hydrogen peroxide. The level of luminescence is unaffected by added hydrogen peroxide or crystalline beef catalase. This of course does not rule out the possibility that organic peroxides may be functional in the system.

In the course of our investigation of the FMN requirement we observed that the purified enzyme without added FMN still emitted appreciable (ca. 5% max.) light when DPNH was added. Microbiological assays using *Lactobacillus casei* confirmed the presence of flavin in the enzyme, and numerous unsuccessful attempts were made to separate it from the enzyme. Irradiation at 5° C with a Keese lamp produced a marked effect in this respect. Figure 2 illustrates the type of result obtained. During an initial period of irradiation added FMN does not give additional stimulation. With continued irradiation, however, the stimulation by FMN rapidly increases until the requirement may be considered essentially absolute.

The more striking effect of irradiation is illustrated in Fig. 3. Samples of the enzyme were removed at intervals and assayed. Both with

and without added FMN the total activity of the enzyme at first increases and subsequently declines with continued irradiation. It was thought that this effect might be due to the destruction of riboflavin,

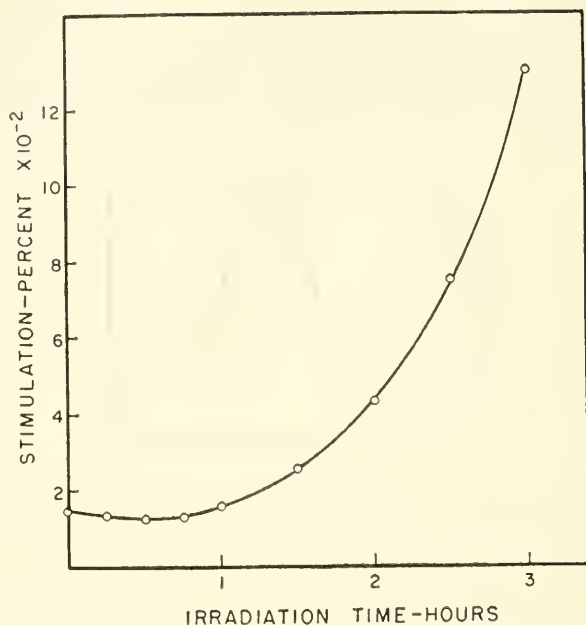


FIG. 2. Stimulation of luminescence by added FMN, using enzyme irradiated for time intervals indicated.

which is a potent inhibitor of the system (Table II). This was shown not to be true when it was found that the stimulatory factor could be produced by irradiation of the 20–30%  $(\text{NH}_4)_2\text{SO}_4$  fraction, which itself had no inhibitory effect and no luciferase activity. The relation-

TABLE II  
Inhibitors of Bacterial Luminescence *in Vitro*

Inhibitor	Concentration, molar	Inhibition, %
Riboflavin	$2 \times 10^{-6}$	43
<i>p</i> -Chloro Hg benzoate	$4 \times 10^{-6}$	58
KCN	$1 \times 10^{-4}$	47
Versene	$7 \times 10^{-4}$	47

ship between irradiation dosage and degree of stimulation when an irradiated 20–30%  $(\text{NH}_4)_2\text{SO}_4$  fraction is added to the reaction mixture is shown in Fig. 4.

Studies concerning the utilization of this factor (McElroy, Hastings, Sonnenfeld, and Coulombre, 1954) indicated that it is destroyed during luminescence and could therefore be considered as bacterial luciferin, analogous to *Cypridina* and firefly luciferins.

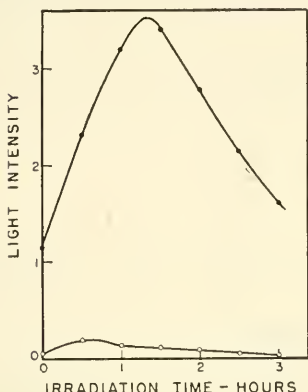


FIG. 3. Effect of irradiation dosage upon activity of enzyme fraction, both with and without added FMN.

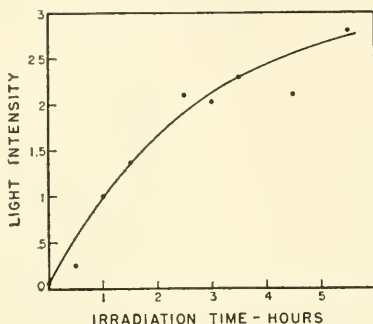


FIG. 4. Effect of ultraviolet dosage upon stimulation of luminescence by 20–30%  $(\text{NH}_4)_2\text{SO}_4$  fraction.

Since long-chain aldehydes will substitute for the factor produced by ultraviolet treatment, it seems possible that a photochemical production of aldehyde occurs during irradiation. This possibility has not been investigated. However, it has been found (McElroy and Green, 1954) that the aldehyde is utilized for light production in the same way as is the factor derived from irradiation. The total light emitted by a reaction mixture is proportional to the amount of aldehyde added (Fig. 5). This evidence is not in accord with Strehler's results which indicate (personal communication) that aldehyde acts only by accelerating the luminescent oxidation of  $\text{FMNH}_2$ . It is possible that the different results may be ascribed to the fact that Strehler has used crude acetonized powders rather than a purified enzyme fraction. With the crude powders it is possible that products of the aldehyde reaction may be reconverted into active aldehyde or that

other enzymatic pathways for the utilization of flavin may exist. This aspect of the problem has not been clarified.

It is important to emphasize that aldehyde destruction occurs only under conditions where luminescence appears, i.e., the enzyme preparation will not destroy aldehyde unless both DPNH and FMN are present. We agree, however, that the aldehyde accelerates the oxidation of FMNH<sub>2</sub>. It does not, however, accelerate the oxidation of DPNH. Apparently the rate-limiting step is the reduction of FMN by DPNH.

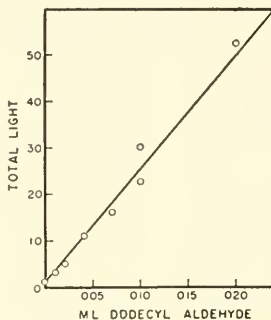


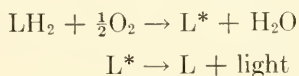
FIG. 5. Effect of varying amount of aldehyde added to reaction mixture upon total light emitted during the reaction (McElroy and Green, unpublished).

The effect of various inhibitors is shown in Table II. The inhibition by *p*-chloro Hg benzoate is reduced from 58% to 17% by  $2 \times 10^{-3}$  *M* glutathione. Although the earlier indication that iron was involved in the system (McElroy, Hastings, Sonnenfeld, and Coulombre, 1954) has not been confirmed, the effect of cyanide and versene suggests that some metal may be functional in the reaction.

In spite of the fact that the peak of bacterial emission is at a shorter wavelength than is riboflavin fluorescence, there is good reason (Strehler, Harvey, Chang, and Cormier, 1954) to suppose that FMN is the light emitter in the bacterial reaction. Moreover, on the basis of its probable role as light emitter, the above authors designate FMN as bacterial luciferin. On the other hand, we have proposed that aldehyde be classified as bacterial luciferin, since it is destroyed during light emission. Although a complete evaluation of the roles of FMN and aldehyde must await additional experimental studies, the question of the criteria by which luciferin may be defined is raised.



DuBois (1887) described luciferin as a substance in the heat stable fraction of *Pholas* extracts which produced light when an unheated dark fraction containing the enzyme was added. McElroy and Harvey (1951) showed that more than one substance could produce this "luciferin" reaction in fireflies. Harvey (1920) defined luciferin extracted from *Cypridina* as "the heat resistant dialyzable substance which takes up oxygen and oxidizes with light production in the presence of . . . luciferase." Although he emphasized the chemical dissimilarities of luciferins extracted from different organisms, *Cypridina* luciferin serves as a model for the general mechanism of bioluminescent reactions, where the overall reaction is written:



Luciferin in this scheme ( $\text{LH}_2$ ) embraces at least two features: (1) it is (or forms) the light-emitting molecule, and (2) it is the reduced compound which is split in the terminal reaction with oxygen, releasing sufficient energy for light emission.


Although no compound with a completely analogous role has yet been demonstrated in either the firefly or bacterial systems, the essential feature of the above scheme is the designation of luciferin as the substrate in the terminal oxidation. Studies by McElroy and colleagues indicate that in the firefly system the compound as isolated is more strictly a proluciferin and must undergo preliminary dark reactions, possibly involving energy transfer from ATP, before its final oxidative reaction. Similar reactions may also occur in the bacterial system. In addition, it is possible that luciferin might not be directly involved in light emission. That is to say, the energy from the oxidative split of luciferin might be transferred to and activate another molecule in the system, which would itself be designated as the light emitter.

These ideas are in accord with the fact that it has not been possible to reverse the luminescent oxidation of luciferin to reform active luciferin. That the reaction involves a rather drastic split of luciferin might be expected on energetic grounds, since energy sufficient for the emission of a quantum of light is derived from the reaction. It would therefore be expected that, if the general mechanism of bioluminescence involves the irreversible oxidation of luciferin, the total

light in the isolated system would be proportional to the amount of luciferin present, analogous to the situation in *Cypridina* (Chase, 1948) and the firefly (McElroy, 1951). This has been found to be the case for the long-chain aldehyde in bacterial luminescence.

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## Inhibition and Activation of Intracellular Luminescence\*

FRANK H. JOHNSON

Department of Biology, Princeton University, Princeton, New Jersey

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For the purposes of this discussion we may define intracellular luminescence as visible light emission resulting from metabolic processes within intact, living cells, such as bacteria, independently of nervous or other physiological mechanisms that regulate the luminescence of tissues and organs in complex organisms, such as the firefly. An understanding of how various factors operate to inhibit or to activate intracellular luminescence is obviously an important aspect of the total problem of bioluminescence in particular. Of no less importance are the contributions that advances in the understanding of this particular process have made, or may make, toward the understanding of the general problem of inhibition and activation in various other biological processes, both relatively simple and highly complicated. It seems appropriate, therefore, to consider both these aspects, and in the discussions which follow, certain principles or concepts, which were either first recognized clearly, or whose establishment has been especially aided through studies of bioluminescence, are listed, together with a few examples indicating the applicability of these concepts to processes other than luminescence.

Intracellular luminescence is limited first of all, of course, by genetically determined potentialities: the catalytic machinery as well as other factors essential for light emission must be present. Among luminous bacteria, mutant strains that emit no visible light, or that exhibit

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various degrees of brightness as compared to the parent strain under similar conditions, have long been known (Beijerinck, 1912, 1915) and have been studied further in recent years (Doudoroff, 1942; Giese, 1943; McElroy and Farghaly, 1948; Miller, Farghaly, and McElroy, 1949; McElroy and Friedman, 1951).

With the genetic potentiality for luminescence, the intrinsic brightness, i.e., the average intensity of light emitted per cell, may vary enormously at different stages of the growth cycle, or with environ-

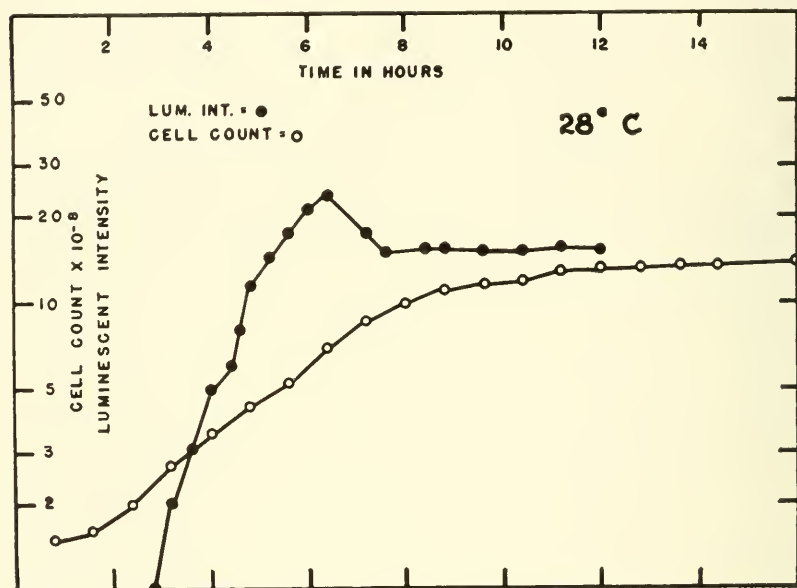


FIG. 1. Rate of increase in cell numbers and in luminescence intensity during aerobic growth of a species of luminous bacteria in 3% NaCl-nutrient broth at 28° C. (After Baylor, 1949.)

mental factors such as temperature of incubation and composition of the medium. Beijerinck (1915) noted that, at relatively high temperatures, growth of *Photobacterium splendidum* will take place, accompanied by very little luminescence. When dark cultures, grown at above optimal temperatures, are placed at a lower temperature favorable for luminescence, light does not immediately appear, although subcultures grow and luminesce at the lower temperature. Along with these studies Beijerinck made the interesting observation

that a suspension of brightly luminescent cells could be momentarily exposed to a temperature high enough practically to extinguish the light, followed by a more or less complete recovery of luminescence on cooling. Much later, this phenomenon was independently rediscovered (Johnson, Brown, and Marsland, 1942) and provided a key to the interpretation of certain temperature-pressure relationships of luminescence as discussed presently.

Quantitative studies on the intensity of luminescence during growth

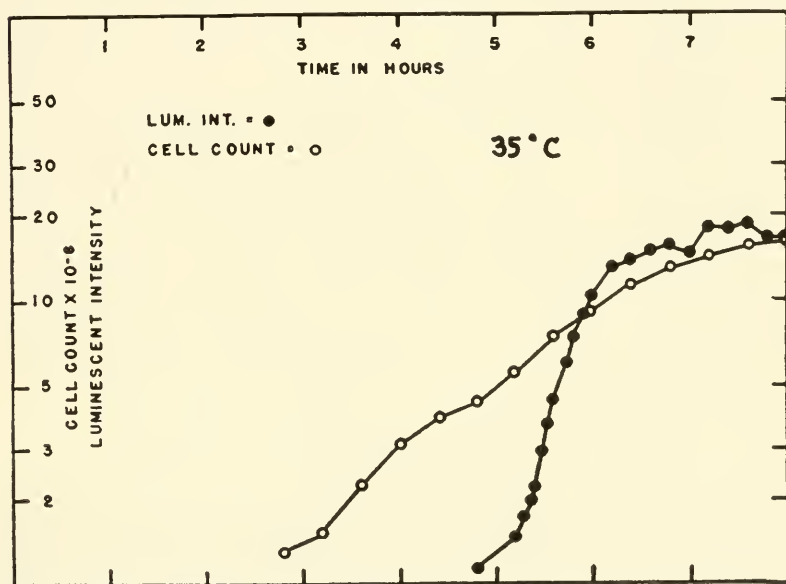


FIG. 2. Rate of increase in cell numbers and in luminescence intensity during aerobic growth of a species of luminous bacteria (same as Fig. 1) in nutrient broth at 35° C. (After Baylor, 1949.)

of a luminescent species at different temperatures have shown that the rate of development of luminescence may differ markedly from the rate of production of cells (Figs. 1 and 2; Baylor, 1949). With other things the same, the temperature coefficient for the overall process of growth and cell division is evidently different from the temperature coefficient for the overall process of the production and functioning of the luminescent system. As a result, the intrinsic brightness per cell may vary many-fold within a growth period of only a few hours.

At a given temperature, the composition of the medium, both with respect to inorganic salts and nutrient constituents, can also profoundly modify the expression of genetic potentialities for luminescence. With minimal amounts (about 0.5%) of NaCl to permit growth of *A. fischeri* in nutrient broth, no visible luminescence occurs (Warren, 1945; Johnson, 1947; Farghaly, 1950), although subcultures in approximately isotonic (3%) NaCl luminesce normally. Complicated

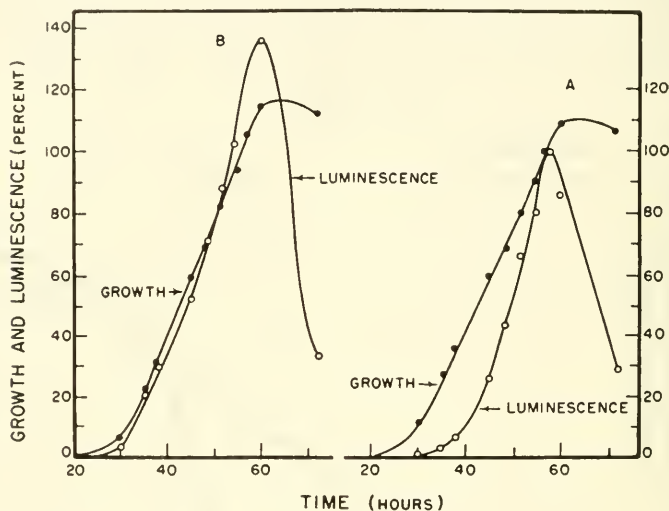


FIG. 3. Rate of growth and of increase in luminescence intensity of *A. fischeri* on a basal medium consisting of inorganic salts plus glycerol (A), and on the same medium with the addition of 3 micrograms of methionine per ml (B). The points on the ordinate are expressed in terms of per cent, with the highest value of luminescence and the corresponding amount of growth on the basal medium without the added methionine arbitrarily taken as 100%. (After Farghaly, 1950.)

relationships have been observed between the luminescence of cultures and the types as well as concentrations of salts in the medium (reviewed by Harvey, 1940, 1952). The same is true of specific nutritive substances, as shown in quantitative studies by Farghaly (1950); for example, the addition of small amounts of methionine to a basic medium practically eliminates the lag in light production as compared to growth (Fig. 3).

Among different species of luminous bacteria, the maximum bright-



ness of a culture, under the most favorable conditions known, varies widely. Psychrophilic, marine species, such as *Photobacterium phosphoreum*, exhibit perhaps the most brilliant luminescence. The luminescence of certain mesophilic, marine species, such as *Photobacterium splendidum* or its very close relative, *A. harveyi* (which should perhaps be considered a subspecies and designated "*Photobacterium splendidum harveyi*") is greatly enhanced by the addition of fish extract to the medium. The significance of the fish extract is not yet known, but the occurrence in fish of vitamin A in the form of the palmitate suggests a possible interpretation, viz., that of providing a source of palmitic aldehyde, very small concentrations of which have recently been found to increase by more than 100-fold the intensity of luminescence in certain extracts of luminous bacteria (Strehler, 1953; Cormier and Strehler, 1953).

Turning now to the problem of inhibition and activation of luminescence in "mature" cells, after they have grown and developed their light-emitting system, the following list includes some of the chief principles that are more or less of general interest. Since the evidence and theory are discussed at length elsewhere (Johnson, Eyring, and Polissar, 1954), they need not be considered in detail here.

### Reversible Denaturation of Intracellular Enzymes

Intracellular luminescence, like essentially all biological processes, exhibits an optimum temperature, or temperature for maximum, over-all reaction rate. The actual temperature varies somewhat among different species, and within a single species it may be reversibly raised or lowered by physical and chemical changes in the environment of the cells. Under given conditions, a reaction of fundamental importance in determining the optimum temperature, and in part the temperature activity curve, is the reversible thermal denaturation of an essential enzyme. Qualitative evidence for this reaction resides in the ready reversibility, by cooling, of the diminution in luminescence intensity during momentary exposures to temperatures well above the normal optimum. Analyses of quantitative data, relating the amount of reversible diminution in intensity to various temperatures above the optimum, indicate that a single reaction, characterized by the high heat and entropy typical of protein denaturation, is primarily

responsible for the thermal diminution. The rapidity of the changes in intensity, on either heating or cooling, is indicative of a mobile equilibrium. The simplest interpretation is that an equilibrium exists between the native (active) and denatured (inactive) form of an enzyme essential to the overall process of light emission. This equi-

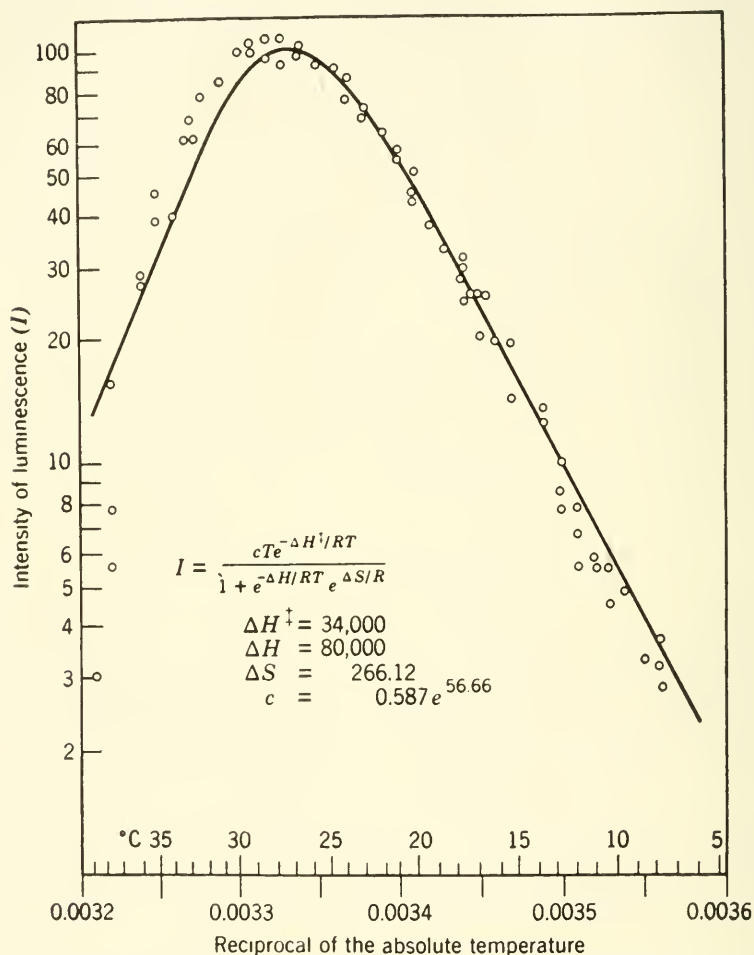


FIG. 4. Influence of temperature on the intensity of luminescence in *A. fischeri*. The smooth curve was calculated in accordance with the equation and constants as given in the figure. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Johnson, Eyring, and Williams, 1942.)

librium reaction, together with the catalytic reaction of a limiting enzyme, are sufficient to account for a major part of the temperature-activity curve. For convenience of discussion, we may designate the equilibrium constant for the reversible denaturation as  $K_1$ , and the specific rate constant of the catalytic reaction as  $k_1$ .

Figure 4 illustrates the observed intensity (by visual photometry) of steady-state luminescence in a suspension of *A. fischeri* cells during brief exposures to various temperatures, above as well as below the optimum. The solid line is a curve calculated in accordance with the equation and constants given in the figure, assuming only the two reactions, with constants  $K_1$  and  $k_1$ , referred to above. Although the theory is obviously oversimplified, the curve fits the data within the limits of experimental error, except at the highest temperatures where destructive reactions with high-temperature coefficients complicate the simplified picture. With some other species of bacteria, it has not proved possible to describe corresponding data with the same accuracy, showing again that the theory is oversimplified in not including additional reactions which appreciably influence the quantitative variation of the overall process with temperature.

Among other processes, the simplified theory describes with considerable accuracy the rate of reproduction of *Escherichia coli* as a function of temperature, from somewhat above to well below the normal optimum of 37° to 39° under the conditions involved (Johnson and Lewin, 1946). Bacteriostasis occurs at about 45° C, but growth is immediately resumed on cooling to 37° C. Analysis of the data indicates that more than one equilibrium reaction is involved in the reversible bacteriostasis at high temperatures, although most of the temperature-activity curve can be accounted for on the same basis as that of bacterial luminescence.

### Volume Changes of Activation ( $\Delta V^\ddagger$ ) in Enzyme-Catalyzed Reactions

Reactions involving only small molecules are not likely to be accompanied by volume changes of activation exceeding a few cubic centimeters per mole (cf. Stearn and Eyring, 1941). With large molecules, such as proteins and enzymes, there is the possibility of large volume changes of activation, of the order of 50 to 100 cc per mole, depend-

ing upon the mechanism of reaction. When volume changes of this magnitude occur, the reaction rate constant is markedly affected by moderately increased hydrostatic pressures of a few hundred atmospheres. The steady-state luminescence of *P. phosphoreum* at a low temperature, where  $K_1$  is negligible, is reversibly reduced by increased

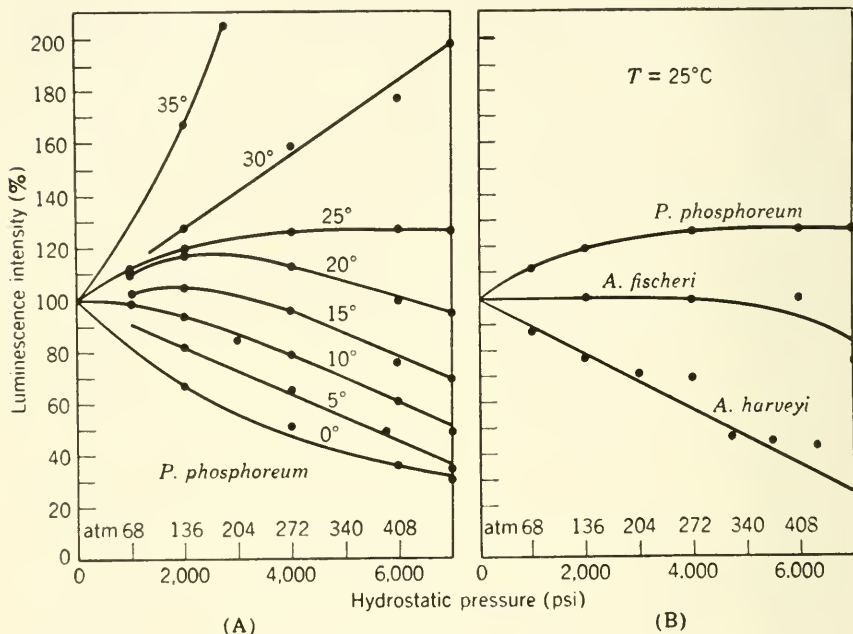


FIG. 5. (A) Intensity of luminescence of *P. phosphoreum* as a function of pressure at different temperatures. The intensity at normal pressure is arbitrarily taken equal to 100 at each temperature in order to show the per cent change in intensity with change in pressure. (B) Influence of increased pressure on the luminescence of three different species of bacteria at 25°C. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Brown, Johnson, and Marsland, 1942.)

pressure (Fig. 5A). The data indicate that the reactions leading to light emission proceed with a net volume increase of activation amounting to about 50 cc per mole at 0° C.

There are, as yet, very few data pertaining to pressure effects on well-defined, purified enzyme systems, for comparison to the luminescence data. Studies with crystallized chymotrypsin and crystallized

trypsin, however, have shown that the value of  $\Delta V^\ddagger$  in the hydrolysis of synthetic peptides varies with the substrate, and may be negative or zero (Werbin and McLaren, 1951a,b).

The observed effect of pressure on the steady-state level of luminescence becomes less as the temperature is raised toward the optimum, and at still higher temperatures the level increases, rather than decreases, under pressure. The family of curves in Fig. 5A resembles a family of curves obtained by Professor D. E. Brown (cf. Johnson, Eyring and Polissar, 1954) for the tension of auricle muscle as a function of temperature and pressure.

Figure 5B shows that the observed effect of pressure at a given temperature depends upon the specific biological system involved. The three species represented in this figure normally exhibit different temperature optima for luminescence, i.e., around 20° in *P. phosphoreum*, 26° in *A. fischeri*, and 30° C in *A. harveyi*. In each case, the effect of pressure is in accordance with what one would expect on the basis that pressure diminishes the intensity of luminescence at temperatures below that of the specific optimum and augments the intensity at temperatures above the specific optimum, with little effect at the optimum. A pressure-temperature relationship of this kind was first recognized by Brown (1934; 1934-35) in studies with muscle.

### Volume Change of Reaction ( $\Delta V$ ) in Reversible Denaturation

The opposite effect of pressure at high and low temperatures, respectively, as illustrated in Fig. 5A, shows that the limiting reactions are not the same at these different temperatures. Since the increase in steady-state luminescence under pressure becomes greater as  $K_1$  of the reversible denaturation becomes greater with rise in temperature, the simplest interpretation is that pressure causes a partial reversal of the denaturation of the enzyme involved. In other words, the equilibrium change from native to reversibly denatured forms of the enzyme is accompanied by a volume increase of reaction. The data indicate that the value of  $\Delta V$  in this reaction amounts to about 65 cc per mole at 35° C.

If we assume as before that there are two reactions primarily limiting the overall luminescent reaction, viz., the catalytic reaction of a limiting enzyme and the reversible denaturation equilibrium, tem-

perature activity curves can be computed for various pressures. In order to obtain a close fit to the data, however, it is necessary to take into account a temperature dependence of  $\Delta V^\ddagger$  and of  $\Delta V$  respectively (Fig. 6), showing again that the simple theory does not include all the reactions that are of importance. It is interesting to note (Fig. 6) that the maximum intensity of luminescence remains practically the same at different pressures, but the temperature at which the maxi-

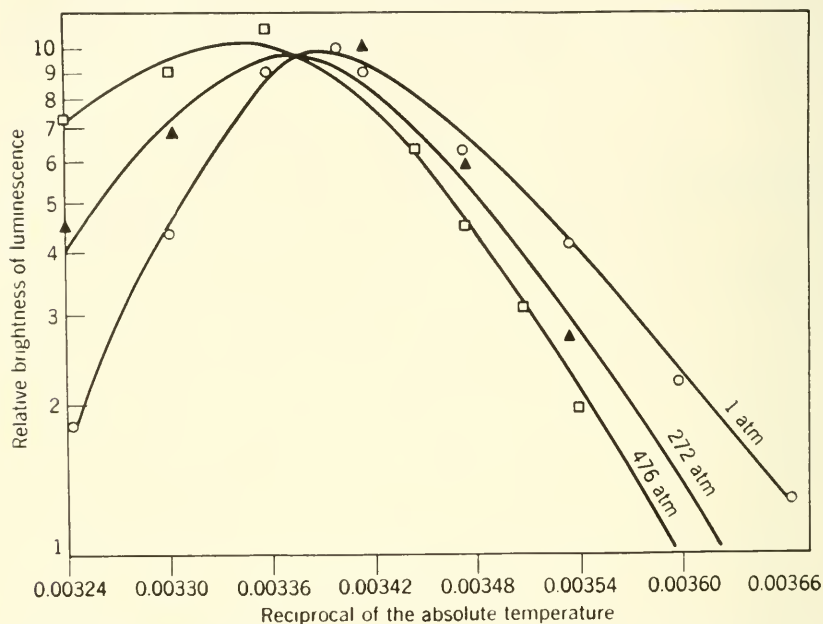


FIG. 6. The brightness of luminescence in *P. phosphoreum* as a function of temperature at three different hydrostatic pressures. The points represent data from experiments by Brown, Johnson, and Marsland (1942). The smooth curves were calculated by Eyring and Magee (1942). (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons.)

imum intensity occurs is higher under increased than at atmospheric pressure.

Although data pertaining to the influence of pressure on the reversible denaturation of isolated enzyme systems are still scarce, there is evidence that highly purified trypsin, with casein as substrate, undergoes a reversible, thermal denaturation accompanied by a considerable volume increase of reaction (Fraser and Johnson, 1951).



## Volume Changes of Activation in Irreversible Protein Denaturation

At temperatures above the normal optimum, the luminescent system is destroyed at rates that increase very rapidly with rise in temperature. These rates have the characteristics of a first order reaction, and their increase with temperature resembles the thermal denaturation of proteins. At a given temperature, however, the rate decreases with rise in pressure (Fig. 7) by an amount indicative of a volume increase of activation of about 70 cc per mole. Thus, it appears that pressure not only acts to reverse the equilibrium change from native to denatured states of an essential enzyme, as discussed above, but also pressure retards the rate of thermal destruction.

When these interpretations, based entirely on kinetic data pertaining to intracellular luminescence, were first expressed, they had no parallel in previous experiments with isolated proteins. Subsequently, it has been shown that the thermal denaturation of highly purified human serum globulin is retarded by pressures up to 10,000 psi (Johnson and Campbell, 1945; 1946), and likewise, tobacco mosaic virus (Johnson, Baylor, and Fraser, 1948). The inactivation of bacteriophage T5 at 68° C (Foster, Johnson, and Miller, 1949) and the disinfection of *Bacillus subtilis* spores at 92.5° to 93.6° C (Johnson and ZoBell, 1949a) are also retarded by increased pressure. The heat inactivation of bacteriophage T7 at 60° C, however, is accelerated under pressure. Moreover, there is essentially no effect of pressure on the salicylate denaturation of methemoglobin (Schlegel and Johnson, 1949), so it is not a general rule that pressures up to about 10,000 psi retard protein denaturation at increased temperatures or in the presence of denaturants.

## Promotion of Reversible Protein Denaturation by Narcotics

The sensitivity of luminescence to narcotics has long been known. In 1672, Robert Boyle reported that the light of "shining flesh" was quickly extinguished by "pouring on it a little pure spirit of wine." Modern studies of the temperature relationships in the inhibition of bacterial luminescence by alcohol, urethan, or other members of the lipid-soluble group of narcotics have provided evidence that the mechanism of their physiological effect involves, in part, promotion

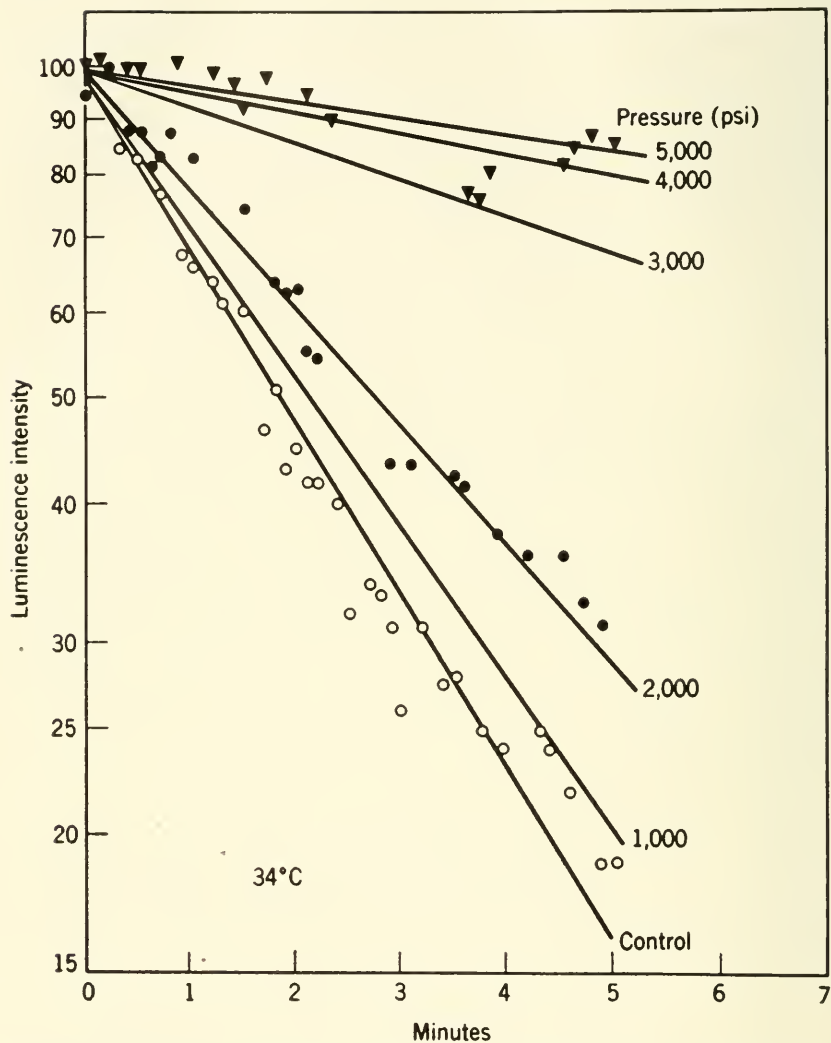


FIG. 7. The influence of hydrostatic pressure on the relative rate of destruction of the luminescent system of *P. phosphoreum* suspended in phosphate-buffered sodium chloride of neutral pH at 34° C. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Johnson *et al.*, 1945.)

of the reversible thermal denaturation of an essential enzyme. In effect, the drug combines with the reversibly denatured form, and the amount of denatured enzyme then becomes significant at a lower

temperature. Thus, with alcohol, for example, the inhibition caused by a given drug concentration increases with rise in temperature (Fig. 8). Stimulatory effects of low concentrations of the drug at low temperatures, as seen in Fig. 8 or encountered under other conditions, must involve other reactions.

Two interesting results of the denaturation-promoting mechanism of action are that, in the presence of alcohol, (1) the measured activation energy for luminescence decreases, and (2) the temperature of

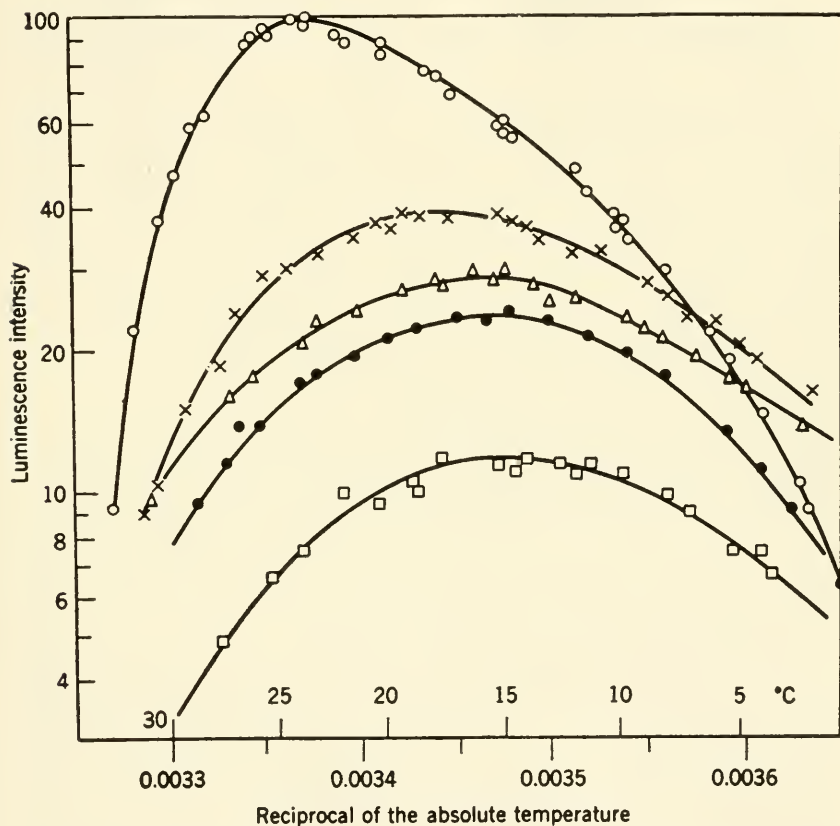


FIG. 8. The influence of temperature on the inhibition of luminescence in *P. phosphoreum* by different concentrations of ethyl alcohol at neutral pH. Alcohol concentrations from uppermost to lowermost curve: 0, 0.4, 0.5, 0.6, 0.8 M. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Johnson *et al.*, 1945.)

maximum luminescence intensity is lowered. Similar effects have been obtained with the urethan inhibition of oxygen consumption and methylene blue reduction by *Rhizobium trifolii* (Koffler, Johnson, and Wilson, 1947). The action of higher members of a homologous series of carbamates on luminescence, however, apparently involves a mechanism different from the one just discussed (Johnson, Flagler, Simpson, and McGeer, 1951).

### Reversal of Narcosis by Increased Hydrostatic Pressure

According to the theory briefly described above, narcotics such as alcohol and urethan promote a reversible denaturation that involves a large volume increase of reaction in going from the initial (native) state to the final (denatured) state of an essential enzyme. It is not surprising, on this basis, to find that the inhibition caused by these drugs is partially counteracted by increased pressure. Figure 9 illustrates the results of applying hydrostatic pressure to aliquot portions of a suspension of luminous bacteria containing different concentrations of alcohol, at the normal optimum temperature where pressure has little effect on the intensity of luminescence in the absence of the drug. At low concentrations of alcohol, the inhibition is virtually abolished by pressure. With higher concentrations, the effects of pressure are more complicated, possibly because of multiple equilibria involved in the action of alcohol (*vide infra*), but in each case the amount of inhibition is reduced by pressure. It is interesting to note also that the "optimum pressure" in the presence of alcohol is reversibly shifted by as much as several thousand pounds per square inch, depending upon the concentration of alcohol.

Although the narcosis of animals is a much more complicated phenomenon than the inhibition of bacterial luminescence, the mechanisms involved at the molecular level are not fundamentally different. It is not surprising, therefore, that salamander and frog larvae, when narcotized in water containing appropriate concentrations of alcohol or urethan, recover their activity and swim in apparently normal manner when the pressure is increased from atmospheric up to between 2000 and 5000 psi (Johnson and Flagler, 1951). Moreover, observations made thus far indicate a close correlation between the action of pressure on the inhibition of bacterial luminescence and on the

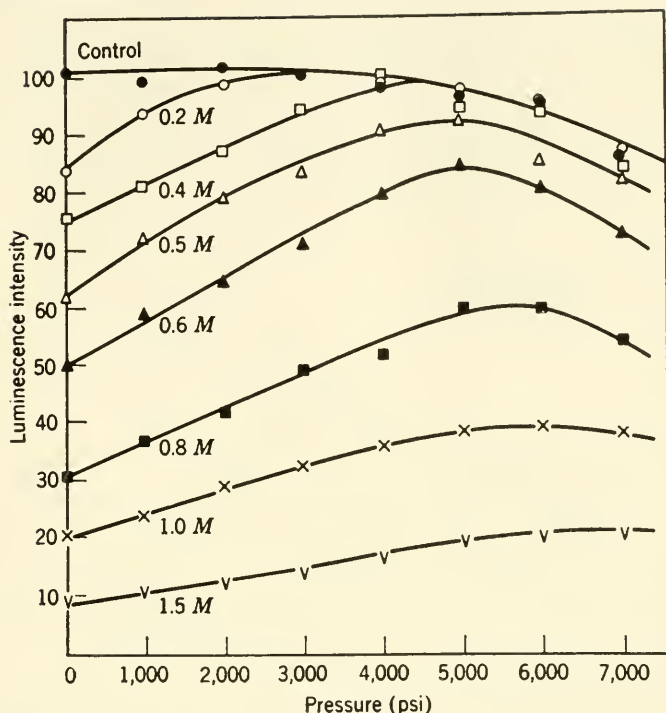


FIG. 9. Luminescence intensity of *P. phosphoreum* as a function of hydrostatic pressure at 17.5° C, in the presence of various concentrations of alcohol in the phosphate-buffered sodium chloride solution, pH 7, of the suspending medium. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Johnson *et al.*, 1945.)

narcosis of these aquatic animals, respectively, by various drugs, i.e., the effects of different drugs on both luminescence and narcosis exhibit varying degrees of sensitivity to pressure that are at least qualitatively the same in the two phenomena.

### Multiple Equilibria in Reversible Effects of Narcotics

At a given temperature and pressure, the relation between drug concentration and amount of inhibition of luminescence by alcohol or urethan often conforms rather closely to the relationship which would be expected if the drug entered into a single equilibrium combination with the enzyme affected. The relationship should be

linear, when plotted in the manner illustrated in Fig. 10, the slope of the line indicating the ratio of drug to enzyme molecules in the equilibrium established. In Fig. 10, it is evident that the relationship is not linear throughout a wide range of drug concentrations, for the slope increases at the higher concentrations. Moreover, the numerical

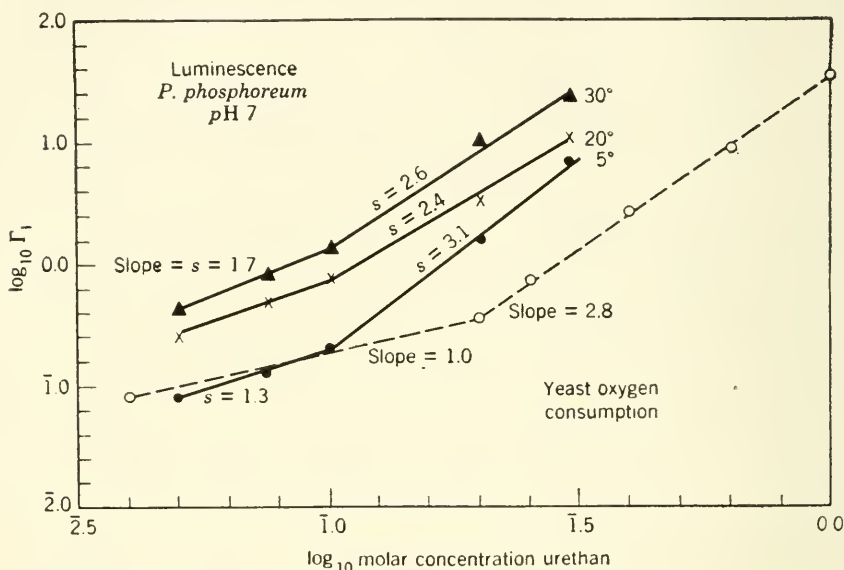


FIG. 10. Relation between concentration of urethan (abscissa) and amount of inhibition (ordinate) of luminescence in *P. phosphoreum* at 5°, 20°, and 30° C, respectively. The symbol  $\Gamma_1$  for inhibition represents  $[(I_c/I_w) - 1]$ , where  $I_c$  is the intensity of luminescence in a control suspension of bacteria, and  $I_w$  is the intensity in a corresponding suspension containing a given concentration of the drug. (Data of Johnson *et al.*, 1945.) The broken line represents data replotted from Fisher and Stearn (1942) concerning the urethan inhibition of oxygen consumption in yeast. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons.)

values of the slopes are not integers, and they change with temperature. They also change with pressure. It follows that more than a single equilibrium is involved in the total effect, and the measured slopes give only the average ratios of the combining molecules.

Figure 10 indicates a similarity in the action of urethan on bacterial luminescence and on yeast respiration. The data on the latter process were interpreted to mean that two different enzyme systems are af-



fected (Fisher and Stearn, 1942). The same results could occur, however, if two or more different equilibria, characterized by different ratios of combining molecules, and very likely also by different heats and entropies of reaction, were established with the same enzyme system. In living cells it is difficult to distinguish between two such possibilities. Action of the drug through multiple equilibrium reactions with a single enzyme is the simpler explanation, however, and there is convincing evidence that urethan catalyzes the thermal denaturation of tobacco mosaic virus through more than a single reaction with this protein (*vide infra*). The denaturation of tobacco mosaic virus in urea at 0° to 40° C also involves multiple reactions between urea and the protein (Lauffer, 1943).

### Catalysis of Irreversible Protein Denaturation by Narcotics

On general considerations it would appear likely that the same drugs which promote a reversible denaturation of enzymes or other proteins would also under appropriate conditions be found to promote, or catalyze, an irreversible denaturation of the same proteins. Urethan, in fact, has been shown to promote the denaturation of egg albumin and serum proteins at room temperature (Hopkins, 1930). In concentrations of physiological interest, urethan catalyzes the destruction of the bacterial luminescent system, at a rate dependent upon the concentration of the drug at a given temperature (Fig. 11). As a result, the initial inhibition, just after adding the drug, increases progressively with time. There are numerous other instances of such a dual action of a drug, which is in marked contrast to the action of others, e.g., of sulfanilamide on bacterial luminescence, wherein the inhibition remains essentially constant with time. The inhibition of respiration of rat brain slices by phenobarbital, for example, indicates a dual action similar to that of urethan on bacterial luminescence (cf. analysis of the data of Jowett (1938) in Johnson, Eyring, and Polissar, 1954).

### Pressure Retardation of Narcotic-Catalyzed Denaturation

On the basis of the foregoing observations, it would be expected that the irreversible denaturation of one or more enzymes essential to bacterial luminescence, in the presence of certain narcotics, would be

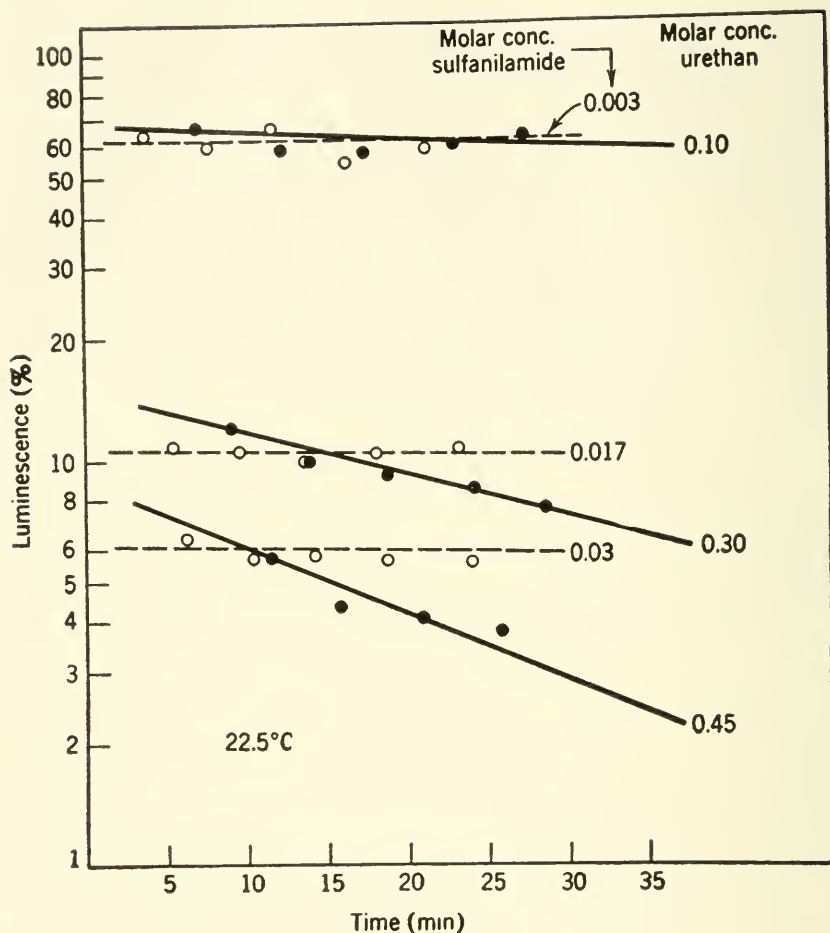


FIG. 11. The time course of the inhibition of luminescence in *P. phosphoreum* at 22.5° C by sulfanilamide (broken lines) and urethan (solid lines), respectively, in concentrations that cause initially similar diminutions of intensity. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Johnson *et al.*, 1945.)

retarded by pressure. Data are not yet available in this regard. It has been shown, however, that pressures of the order of 10,000 psi markedly retard the precipitation of human serum globulin at 65° C in the presence of small concentrations of ethyl alcohol which accelerate the rate of precipitation (Johnson and Campbell, 1946). Similarly,

small concentrations of urethan catalyze the precipitation of tobacco mosaic virus at 68.8° C in a manner that is retarded by pressure (Fraser, Johnson, and Baker, 1949). The same is true of the thermal disinfection of bacteriophage T5 in the presence of urethan (Foster, Johnson, and Miller, 1949). Finally, the accelerated disinfection of *B. subtilis* spores in the presence of urethan is retarded by pressure (Johnson and ZoBell, 1949b). Although further examples of this sort of phenomenon may be expected it is not to be looked upon as a general rule, inasmuch as effects of pressure depend upon the mechanism of the reaction, and among a variety of drugs as well as of experimental conditions (temperature, pH, dissolved salts, etc.) the mechanisms of denaturation, even with the same protein, may be expected to differ in detail.

### Multiple Reactions in Narcotic-Catalyzed Protein Denaturation

Here again, data with respect to intracellular luminescence are not yet available, but it is reasonable to expect certain similarities to the results that have been obtained with other systems in studies stemming from work on bacterial luminescence. A particularly clear example of multiple reactions is afforded by the action of urethan in catalyzing the thermal denaturation of tobacco mosaic virus, wherein at least two, and probably three, distinct reactions between urethan and the protein are involved (Fraser, Johnson, and Baker, 1949).

Now, since many of the interpretations discussed above have been based primarily on kinetic data pertaining to the luminescence of intact, living cells, additional evidence is needed before these interpretations can be considered fully convincing. Such need is met in part by finding, and in some instances anticipating, fundamentally similar phenomena in various processes other than luminescence, i.e., denaturation of purified proteins, reactions of purified proteins, activity of purified enzymes, rates of growth and disinfection, and other examples mentioned. Until quite recently, however, it has not been possible to investigate the influence of different factors—temperature, pressure, narcotics, etc.—on bacterial luminescence in cell-free extracts. Strehler's success in obtaining luminescent extracts (Strehler, 1953) has provided a long sought means not only of getting more direct evidence than is possible with intracellular luminescence but also of

identifying biochemically certain components of the luminescent system, as well as of purifying the enzymes involved. Rapid progress has already been made toward the latter objectives (Cormier and Strehler, 1953; McElroy *et al.*, 1953, 1954; Strehler *et al.*, 1954; Strehler and Cormier, 1953).

For the remainder of the present discussion, it is appropriate to dwell on results that have been obtained in a joint study (Strehler and Johnson, 1954) of pressure-temperature-inhibitor relationships of bacterial luminescence in cell-free extracts.

### Kinetics of Bacterial Luminescence *in vitro*

The observations described in the following paragraphs were made with extracts of *A. fischeri* cells, prepared by treating first with cold acetone, and then taking up the dried powder in distilled water, followed by high-speed centrifugation to remove all particulate matter. The supernatant provided a stock enzyme solution which was diluted with phosphate buffer at neutral pH as needed. The addition of flavine mononucleotide (FMN), dihydrodiphosphopyridine nucleotide (DPNH<sub>2</sub>), and decaldehyde in final concentrations of approximately 0.3 microgram, 1 mg, and 7 micrograms per milliliter, respectively, gave a "saturated system" that emitted a bright luminescence over extended periods of time at room temperature.

Figure 12 illustrates the changes in intensity of the saturated system after the sudden application and sudden release, respectively, of hydrostatic pressure at temperatures below, above, and near that of the optimum (about 26° C) of the system used. One curve (16°, Cells), obtained with a suspension of cells of the same species, is included for comparison to the data obtained with the extracts.

Among the several points of interest shown by these data are the following: (1) the changes in luminescence intensity after application of pressure are reversible upon release of pressure, (2) a fairly sudden but transitory increase in intensity ("spike") occurs on sudden application of pressure, and a fairly sudden but transitory decrease ("dip") occurs on sudden release of pressure, (3) the spikes and dips are followed by relatively slow changes to a new steady-state level under increased or at normal pressure, respectively, (4) the new steady-state level is lower, nearly the same, or higher than the initial

level before applying pressure, according to whether the temperature is below, near, or above the normal optimum temperature of the system, and (5) the steady-state level of luminescence intensity after releasing pressure is the same as the steady-state level before applying

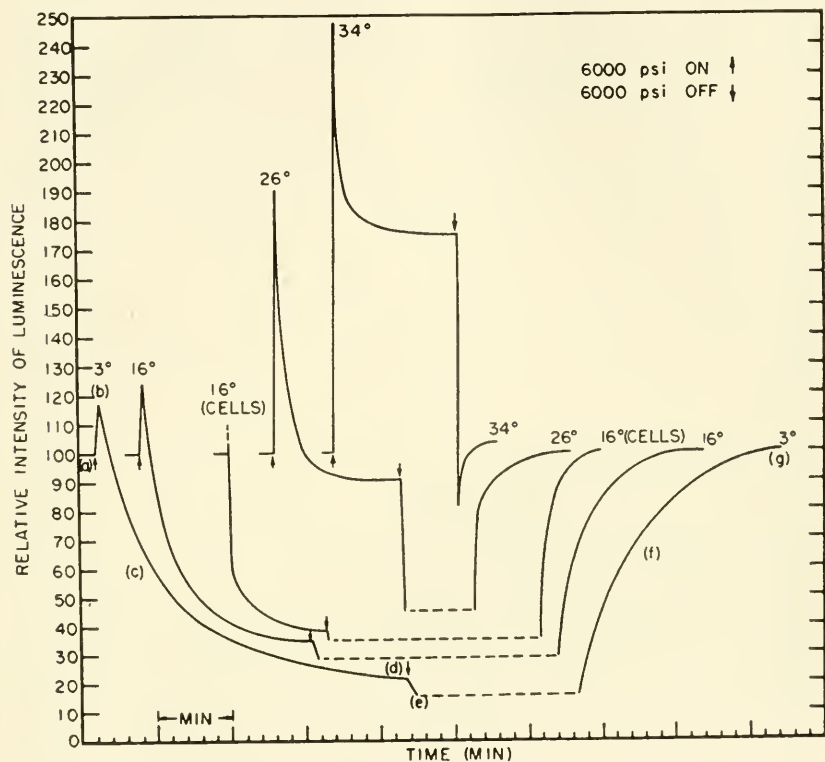


FIG. 12. Influence of pressure at different temperatures on the luminescence of cell-free extracts of *A. fischeri* with added DPNH<sub>2</sub>, FMN, and decaldehyde (saturated system). Arrows pointing upwards indicate the time when pressure was applied; arrows pointing downwards, when it was released. The curves for rising intensity after release of pressure (except the curve for 34° C) have been arbitrarily displaced to the right on the abscissa, to avoid intersecting lines. The intensity at normal pressure, just before raising the pressure, is arbitrarily taken equal to 100 at each temperature, and all other points computed relative to this value. Allowance was made for the amount of decay when it was significant during the periods involved, as at the higher temperatures. The increased pressure was 6000 psi at each temperature except 3° C where it was 6500 psi. Data obtained with intact cells of *A. fischeri* at 16° C are included for purposes of comparison. (After Strehler and Johnson, 1954.)

ing pressure, provided correction is made for the decay of luminescence during the intervening period of time, and at the higher temperatures, provided correction is made also for a rate of thermal inactivation of the system.

Evidence of the transitory spikes and dips had been observed in earlier studies with intact cells (cf. discussion in Johnson, Eyring, and Polissar, 1954), but quantitative records had not been made of them or of the changes in intensity between steady-state levels. Data are not yet available whereby, at various temperatures, quantitative comparisons can be made between spikes and dips in cells and extracts, respectively. It appears that the transitory changes occur more rapidly in the intact cells, as judged by the curves shown for 16° in Fig. 12. The true magnitudes of the spike and dip in cells at 16° C are uncertain because the half-second period of the recording instrument was not fast enough to measure them accurately. Qualitatively, the results obtained with extracts are remarkably similar to those obtained in previous studies with intact cells. Quantitatively, the agreement is close, at the one temperature for which data are presently available, except that the time relations differ, i.e., changes in intensity following changes in pressure take place faster in cells than in the extracts.

Steady-state levels of intensity as a function of pressure at various temperatures are plotted in Fig. 13, from the data of Fig. 12 and similar experiments. For comparison, the pressure-temperature data obtained with cells of *P. phosphoreum* in the initial study of this relationship in luminous bacteria (Brown, Johnson, and Marsland, 1942) are replotted in this figure. Corresponding effects of pressure occur at temperatures a few degrees higher in extracts of *A. fischeri* than in cells of *P. phosphoreum*, a difference that would be logically expected on the basis of the difference in normal optimal temperatures for luminescence in the two species, i.e., the normal optimum is several degrees higher in cells and extracts of *A. fischeri* than in cells of *P. phosphoreum*. As indicated earlier in the discussion, the biological effects of pressure may be expected to be related to the temperature-activity curve of the specific system and conditions involved. Thus, the increase in steady-state levels under pressure becomes progressively greater as the temperature is increased above the normal optimum of the specific system. The most likely interpretation is that



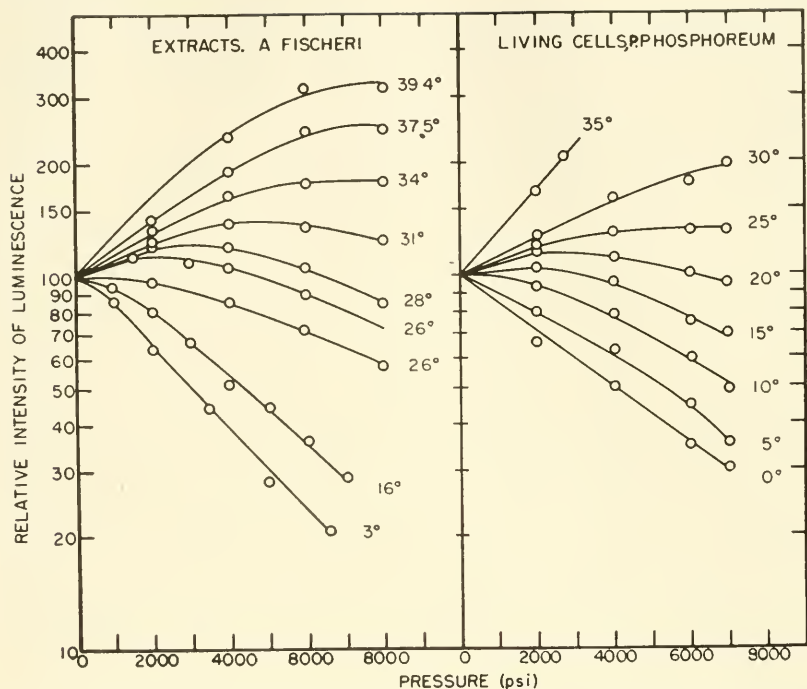


FIG. 13. Steady-state levels of luminescence in the saturated system of *A. fischeri* extracts (left) and in living cells of *P. phosphoreum* (right). The data on the right are replotted from Brown, Johnson, and Marsland (1942). The initial intensity at normal pressure is arbitrarily taken equal to 100 at each temperature, and allowance is made for decay, when significant, in the luminescence of extracts. The lower of the two curves for 26° C was obtained with an enzyme solution that had stood for several days at room temperature. (After Strehler and Johnson, 1954.)

which has already been expressed, namely, that at these relatively high temperatures, pressure acts to reverse an equilibrium change from native to denatured states of an essential enzyme (or enzymes), thereby increasing the overall velocity of the light-emitting reaction. Qualitative evidence for a reversible thermal denaturation of the extracted enzyme system was found by visual observation: diminution in intensity occurs on momentary exposures of luminescent solutions to relatively high temperatures, followed by recovery on cooling. At low temperatures, where the amount of thermally denatured enzyme is negligible, pressure merely reduces the steady-state level.

The straightness of the line for 3° C in the left-hand part of Fig. 13 indicates that a single reaction is primarily affected, and the slope of this line indicates that this reaction proceeds with a volume increase of activation of 86 cc per mole.

With the extracted system, it is possible to interpret in further detail the influence of pressure on the reactions involved in luminescence. Omitting the complications introduced by pressure-sensitive equilibria between native and thermally denatured forms of essential enzymes, and considering only the effects observed at temperatures below the optimum, the evidence favors the view that the large volume increase of activation is associated with the reduction of FMN by  $\text{DPNH}_2$ , and it is for this reason that the steady-state level at low temperatures is reduced by pressure. In the absence of added  $\text{DPNH}_2$ , a rapidly decaying luminescence occurs on addition of reduced FMN to a solution of enzyme plus aldehyde. The rapidity of decay makes it difficult to determine clearly whether or not there is any change under pressure corresponding to the change in steady-state levels of the complete system under pressure. The data clearly reveal, however, that sudden increases and sudden decreases in intensity accompany the sudden application and release, respectively, of pressure during the rapid decay of luminescence. The magnitude of these sudden increases and decreases corresponds to that of the spikes and dips, and careful analyses of the data failed to reveal evidence of changes which could be interpreted as corresponding to effects of pressure on steady states. Thus, it appears that the spikes and dips are associated with the luminescent oxidation of the flavin component, which appears to proceed with a small volume decrease of activation, of the order of  $-10$  cc per mole.

Further interpretations require taking into consideration the influence of pressure on the rate of change in luminescence intensity between two different steady-state levels due to a change in pressure. With the saturated system at temperatures below the normal optimum, the rates of change, after application of pressure, between the spike peaks and the lower steady-state levels, conform to first order kinetics. The same is true for the rates of change, after release of pressure, between the bottoms of the dips and the higher steady-state levels. In Fig. 14 are plotted representative data which show that

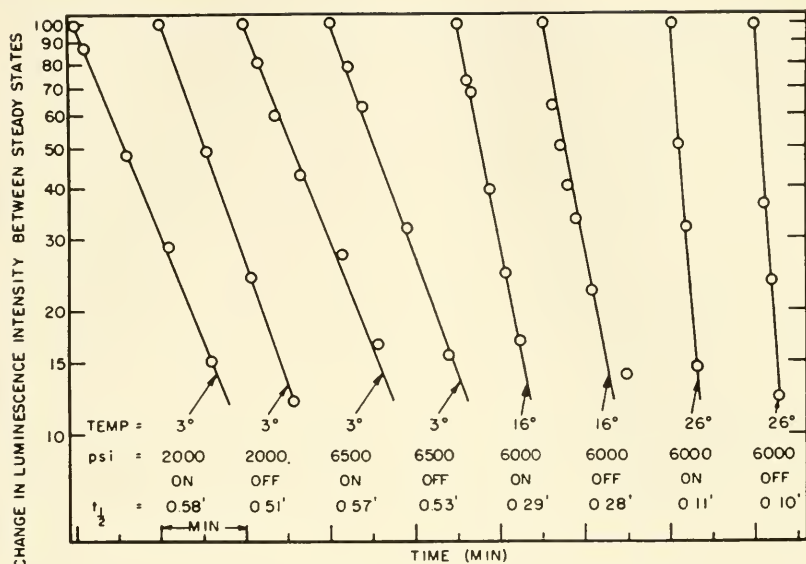
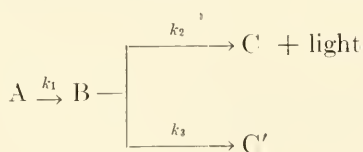


FIG. 14. Rate of change in intensity of luminescence in the saturated system following application (on) and release (off) of pressure at different temperatures. The half time ( $t_{1/2}$ ) for the change between steady states is indicated below the respective lines. The lines, plotted relative to an arbitrary value of 100 at  $t = 0$ , represent the progressive difference in intensity, with time, between the peak of the spike and steady-state level under pressure, and the progressive difference, with time, between the lowest point of the dip and the steady-state level after pressure, respectively.

although the rates increase with rise in temperature, they do not vary significantly with the amount of pressure at a single temperature. The differences in rates under increased as compared to normal pressure are small but perhaps significant.

The following simplified diagram is helpful in picturing the relationship of some of the reactions involved in luminescence of the saturated system at low temperatures:



In this diagram, A stands for  $\text{DPNH}_2 + \text{FMN}$ , B stands for  $\text{DPN} + \text{FMNH}_2$ , and C and C' stand for the products of the luminescent and

of all nonluminescent reactions, respectively, of FMNH<sub>2</sub>. The  $k$ 's stand for specific rate constants of the reactions as indicated.

With DPNH<sub>2</sub> present in excess in the experiments described, its concentration remains essentially constant over short periods of time. When the pressure is changed, the rate constants change immediately, in accordance with the amount of pressure and the respective values of the volume change of activation constants. On the basis of the evidence at hand,  $k_1$  is characterized by a large volume increase of activation, whereby it becomes smaller, and the steady-state intensity therefore lower, under increased pressure. Similarly,  $k_2$  is characterized by a small volume decrease of activation, whereby it becomes slightly larger under pressure than at normal pressure, thus giving rise to transitory spikes and dips. (At elevated temperatures the much greater magnitude of the spikes and dips is presumably due to the relation of denaturation equilibria of one or more enzymes to pressure.) The rate of change between steady states, with change in pressure, is given by the exponential factor in the following equation, derived in line with the theory of consecutive first order reactions discussed elsewhere (Johnson, Eyring, and Polissar, 1954; Strehler and Johnson, 1954):

$$I = bk_2[B] = bk_2 \left\{ [B_0] - \frac{k_1[A]}{k_2 + k_3} \right\} e^{-(k_2 + k_3)t} + \frac{k_1[A]}{k_2 + k_3}$$

In this equation,  $I$  represents the intensity of luminescence,  $b$  is a proportionality constant,  $B_0$  is the initial concentration of B at the time when pressure is applied or released, and the  $k$ 's refer to the reactions as diagrammed above. It will be noted that reactant concentration does not enter into the exponential factor; the rate of change between steady states is  $\exp. - (k_2 + k_3)t$  equals the slopes of the lines in Fig. 14. Since  $k_2$  is sensitive to pressure, whereas  $(k_2 + k_3)$  is not appreciably sensitive to pressure, it follows either (1) that  $k_3$  is very much larger than  $k_2$  or that (2) compensatory changes in  $k_2$  and  $k_3$  occur under pressure. Of these alternatives, the former appears far more probable. Among other reasons, unless  $k_3$  were much larger than  $k_2$ , the quantum efficiency would be extraordinarily high. The nonluminescent, auto-oxidation of reduced FMN probably accounts, in large part, for  $k_3$ .

Although extensive studies concerning the action of inhibitors on the luminescence of bacterial extracts have not been undertaken as yet, exploratory experiments with the saturated system have yielded some interesting results. Thus, sulfanilamide reduces the steady-state level of luminescence, and the amount of inhibition at a given concentration of the drug decreases with rise in temperature, whereas it is only slightly affected with rise in pressure, as in cells. The actual concentration required for a given per cent inhibition in extracts of *A. fischeri*, however, is many times higher than that required in cells of this species, possibly because of the presence, in these extracts, of large amounts of inert material on which the sulfanilamide adsorbs. Ethyl alcohol or urethan also inhibit the steady-state luminescence of extracts, at concentrations that are only 2 or 3 times greater than those required for corresponding per cent inhibitions of cellular luminescence under similar conditions of temperature and hydrostatic pressure. The inhibitory concentrations of alcohol or urethan, however, are considerably higher than those of sulfanilamide, and it is reasonable to suppose that adsorption on inert substances has less effect in altering the concentration initially added to the solution. As in cells, the inhibitory effect of a given concentration of alcohol or urethan on steady-state luminescence increases with rise in temperature and decreases with rise in pressure. These drugs also affect the spikes: at optimum temperature and normal pressure, the spike is markedly higher in the presence of inhibitory concentrations of either drug. Subinhibitory concentrations of alcohol increase the steady-state intensity of luminescence in extracts. Increases in the steady-state intensity of cellular luminescence, in the presence of alcohol under certain conditions, have also been noted (cf. Fig. 8).

The role of the aldehyde remains to be clearly established. In the absence of added aldehyde, the pressure-temperature relationships are different, but the intensity of luminescence is so weak that these relationships have not been determined with a desirable accuracy by means of the methods available in the initial study. The evidence at hand indicates that unless adequate concentrations of aldehyde are present, steady-state levels of luminescence are not significantly lowered by pressure at low temperatures, and the magnitude of the spikes is somewhat less. Thus, the limiting reactions are not the same



in the absence of aldehyde. With the addition of minimal concentrations to cause a fairly bright luminescence, the kinetics of the changing intensity due to pressure is again different. It is difficult at the moment, to arrive at an explicit hypothesis that provides a uniquely probable explanation of these observations.

The correspondence between the pressure-temperature-inhibitor relationships of luminescence in living cells and in the saturated extracted system argues for the fundamental similarity of the luminescent process in cells and in these extracts. Moreover, this correspondence shows that the earlier observations, made with intact cells and interpreted in terms of simple systems, can actually be attributed to the operation of relatively simple systems, rather than to extremely complicated relationships existing in the highly organized chemical environment of living cells, and resembling simple reactions only by chance. Thus, justification is provided for the conclusions reached earlier in regard to intracellular luminescence, and at the same time, for further studies with the living cells, which in a number of respects are more convenient to use than extracts. It is only with extracts, of course, that the biochemistry of the system can be firmly established, and that detailed analysis of the physical chemistry of the system can be made under fully controllable conditions. A great deal can be learned also, however, from studies of the relatively simple process within the immensely complex setting of a living cell. The study of luminescence, *in vivo* as well as *in vitro*, is of general as well as of particular interest.

#### ACKNOWLEDGMENTS

The author is indebted to Professor W. J. Kauzmann for the derivation of the equation on page 290, and for critical discussions of the kinetics of luminescence in bacterial extracts. The views expressed in this paper are those of the author, and they do not necessarily coincide with those of all the authors and co-authors of the papers referred to in this paper.



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## DISCUSSION

**Dr. Kauzmann:** Among a few examples wherein denaturation is accelerated by pressure is the denaturation of ovalbumin in urea at 0° C, which is accelerated by a factor of about 10 on applying 9000 psi (Simpson and Kauzmann, 1953). On the other hand, at 40° C under the same conditions, there is no effect of pressure. One might therefore expect that at still higher temperatures pressure would tend to reverse or retard the denaturing effect of urea, in accordance with the other examples of retardation of protein denaturation by pressure, just discussed by Dr. Johnson.

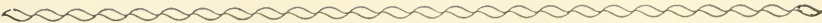
The work of Linderstrøm-Lang's group on the volume changes which occur in the enzymatic hydrolysis of native beta lactoglobulin also has some bearing on this point (Linderstrøm-Lang, 1952; Linderstrøm-Lang and Jacobsen (1941)). These workers have found that during the initial stages of degradation there is a volume decrease amounting to several hundred cubic centimeters per mole. They have ascribed this to a disruption of the native protein structure and have given reasons for believing that this is akin to denaturation by agents such as heat and urea.

**Dr. Mason:** A good deal of evidence, especially with oxidative systems, has demonstrated that many enzymes tend to be aggregated, possibly in a highly ordered way, upon intracellular particulates and that their activities are dependent upon or modified by their order in this aggregation. In considering the relationship between the response of intact bioluminescent cells and extracts of those cells to changes in environmental conditions such as pressure and temperature, is it not possible that particulate suspensions and solutions of enzymes may display qualitatively the same effects, yet be responding to the changes in conditions by essentially different mechanisms?

**Dr. Johnson:** That would seem possible, of course, but it would be a more complicated interpretation of the results discussed. I do not believe that the similarities of the pressure-temperature-inhibitor relationships of luminescence in the extracts to those in living cells are merely coincidental, involving different mechanisms; in my opinion, the evidence favors the view that they are fundamentally the same.







## Physiological Control of Luminescence in Animals

J. A. C. NICOL\*

Marine Biological Laboratory, Plymouth, England

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Luminescence appears to be almost universally subject to some degree of regulation in animals (Harvey, 1953). As an initial approach we can distinguish two main categories of luminescence, namely extrinsic or bacterial, in which the light is produced by bacteria harbored by the animal; and intrinsic, in which photogeny depends upon the animal's own biochemical accomplishment. In either event, overt appearance of light, i.e., light emission to the exterior, is usually controllable.

If luminescence is considered in terms of effector systems, then I think we can concede the following classification: (1) continuous production of light by symbiotic bacteria; (2) discharge of luminous secretion into a surrounding aqueous medium; (3) intracellular luminescence by the animal's photocytes. This is the foundation of my discussion, and in this paper I shall review certain aspects of luminescence from the viewpoint of neuro-effector control.

It would appear that symbiosis with luminescent bacteria is relatively rare. The most interesting and best authenticated instances of symbiosis are encountered in certain marine teleosts, in which luminescent bacteria are harbored in special circumscribed organs. By the use of screening devices the light emitted by the bacteria can be occluded or revealed. A few instances are also known of continuous light emission on the part of the animal, e.g., in pelagic squid *Spirula*, a millipede *Luminodesmus*, the larva of the beetle *Phengodes*, and

\* Guggenheim Canadian Fellow.

eggs and pupae of lampyrids. Apart from these instances, in which control appears to be lacking, some form of regulation of light emission is the rule (Buck, 1948; Harvey, 1952; Davenport *et al.*, 1952).

In the evolution of animals, effectors have preceded neural regulation, as emphasized by Parker (1919). This condition is still existent in two phyla, the Protozoa and Porifera.

### Regulation of Luminescence in Absence of Differentiated Nervous System

The Protozoa are sensitive to a multiplicity of environmental agents, to which they respond mechanically, by changes in body shape, movement of organelles, and chemically, by secretion and by light production. In *Noctiluca*, which has been most studied, luminescence is evoked normally by mechanical stimulation, resulting in a brief flash; and with interrupted induced current the animals flash on the first shock and remain glowing thereafter.

These observations pose several problems, which are not necessarily peculiar to *Noctiluca*, nor to the luminescent reaction. Local and gentle tactile stimulation results in luminescence only in the region of the cell affected. A generalized luminous response, therefore, depends upon agitation of the whole cell, or transmission of excitation. Photogenic granules are believed to lie superficially in the cell. However, propagation of an excitatory wave may well be other than a surface phenomenon; and an analogy is at hand in transmission of excitation across a muscle fiber, the contractile proteins of which lie within. It is, perhaps, relevant that punctured and collapsed *Noctiluca*, with injured membranes, give normal luminescent responses to mechanical and electrical stimuli (Robin and Legros, 1866; Allman, 1872; Massart, 1893; E. B. Harvey, 1917).

Another approach to this problem, full of interest, may be indicated here. There are several metazoan groups in which eggs and larvae are luminescent, preceding or following establishment of innervation. In ctenophores, eggs and early segmenting stages light up when stimulated, either by a mechanical disturbance or an electrical shock. Larvae of the polychaete *Chaetopterus* also luminesce when agitated, but these are metamorphosing trochospheres in which a larval nerv-

ous system is already defined (Peters, 1905; Yatsu, 1912; Enders, 1909).

With the highly sensitive multiplier phototubes now available, the luminescent responses of some of these forms may be amenable to photoelectric recording. They afford additional instances of direct responses to environmental changes before the advent of nervous control and provide experimental material for the study of excitation and effector activity at the cellular level.

### Luminescence and Nerve Net

Many coelenterates, both pelagic and benthic, are luminescent. It is characteristic of these animals that their responses to environmental changes are controlled by a nerve net. This is to a large extent a meshwork of discrete neurones, across which two-way conduction takes place. The quality and magnitude of response are governed by various factors, prominent among which is neural facilitation. This is a selective condition governing behavior by which seriated impulses bring about responses which are out of all proportion to the apparent effect of a single impulse.

Luminescent responses in certain pennatulids will illustrate the functioning of the nerve net. The response in these animals takes the form of a flash of light which sweeps over the surface. In sea pens *Pennatula*, a stimulus applied at any point excites a wave or waves which sweep away from the affected area. Similarly, simultaneous stimulation at the two extremities excites convergent waves. Confirmatory experiments, showing that conduction is nonpolarized, have been carried out on *Renilla*, in which animal it has been shown that complex cuts, producing devious pathways, still allow transmission (Panceri, 1872a; Parker, 1920a, b).

In many coelenterates several impulses are necessary to evoke a response owing to the intervention of facilitation. Harvey noted that *Cavernularia* usually failed to respond to a single shock, but gave a flash after three shocks in rapid succession. In *Renilla* it is found that several shocks are necessary to elicit a luminous wave, and with continued stimulation the consecutive flashes increase progressively in intensity (Fig. 1). Both the aforementioned features (ineffectiveness

of a single pulse, augmentation of intensity in successive responses) are typical of facilitation. Observation shows that transmission is non-decremental, i.e., a wave once initiated, courses over the whole surface of the rachis. There are cogent reasons for believing that facilitation

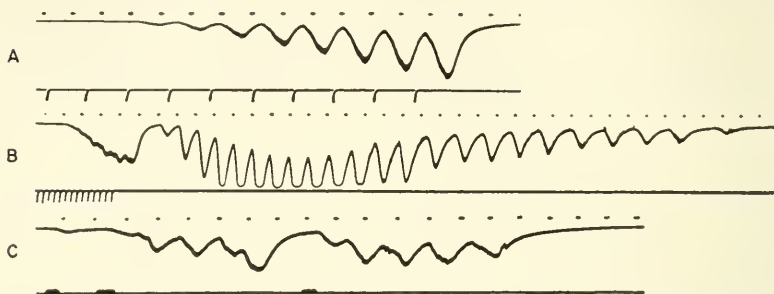


FIG. 1. Luminescent responses of the sea pansy *Renilla*. A, series of responses to a burst of 10 electrical shocks at a frequency of 1 per second. The first response appears after the third shock, and subsequent responses increase progressively in intensity (facilitation). B, responses to a burst of shocks at high frequency (3 per second). Note prolonged after-discharge. C, flashing induced by tactile stimulation. Time scale above, 72 per minute. Recording from the entire rachis. Luminous responses shown as downward deflections of middle trace; electrical stimuli shown on lower line. Photomultiplier + cathode-ray oscilloscope recording.

occurs peripherally, at the neuro-effector junction, and not in the synapses of the nerve net. A single tactile stimulus gives rise to a wave or a series of waves, and this type of response is usually ascribed to facilitation, resulting from a volley of impulses set off by mechanical stimulation of receptors (Fig. 1) (E. N. Harvey, 1917; Pantin, 1935; Buck, 1953, 1955).

Well-defined synapses have been recognized in medusae, and these exhibit nonpolarized conduction (Bozler, 1927). Using contractions of the umbrella as indicative of transmission phenomena, Bullock (1943) finds that the nerve net of scyphomedusae is in a state of permanent facilitation and transmits each impulse. Continued stimulation, however, produces staircase, indicative of neuromuscular facilitation. In the luminescent scyphomedusan *Pelagia noctiluca* tactile and electrical stimulation evokes a glow which may spread in some animals as a wave over the surface of the bell (Panceri, 1872b; Heymans and Moore, 1924; Moore, 1926). By stimulating with con-

denser shocks, I have found that the animal gives a flash to each stimulus (Fig. 2). The total duration of a local flash is 3 seconds, and time taken to reach maximal height occupies 0.2 second. There is no evidence for facilitation in my records, since each shock brings up

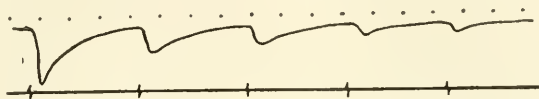


FIG. 2. Flashes of the scyphomedusan *Pelagia noctiluca* to electrical stimulation (condenser shocks). Burst of 5 shocks at about 12 per minute. Time scale 1 per second. Stimuli added to this record represent number but not absolute position of pulses.

a response, and the intensity of consecutive flashes decreases in an exponential fashion (Figs. 2 and 3). Indeed, this progressive decay of photogeny is the most obvious feature of the records and may well be obscuring facilitation in the transmission system.

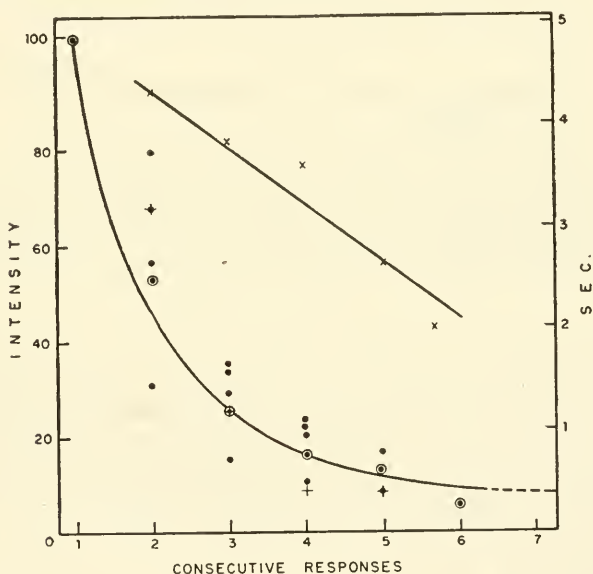


FIG. 3. Fatigue of consecutive responses in *Pelagia noctiluca*. The animal was stimulated with a burst of stimuli (condenser shocks) at a frequency of 1 per second. Upper curve, flash duration (ordinates, on right, in seconds). Lower curve, decrease in intensity of flash and in total light emitted in successive flashes.  $\odot$  mean flash intensity.  $+$  integration of total light emitted during a flash.

Only limited information is available for the nerve net of ctenophores. On tactile stimulation, light appears under the combs, and conductile pathways are likewise restricted to these areas. Nervous transmission along the meridians is evidently unpolarized, since a luminescent wave is capable of oral or aboral passage (Panceri, 1872b; Peters, 1905; Moore, 1924, 1926). With electrical stimulation, a flash appears on each stimulus after the first. These flashes are of very brief duration, about 0.2 second, and summate at frequencies above 5 per second (Fig. 4). Facilitation of response is also clearly recognizable at slow frequencies of stimulation, the first few responses increasing progressively in height to plateau level, after which fatigue sets in. With repetitive stimulation, rhythmic flashing is also induced (*Beroë*, Fig. 4).

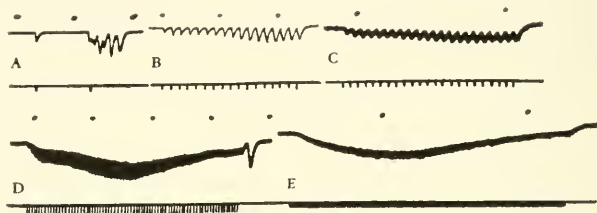


FIG. 4. Responses of the ctenophore *Beroë* to electrical stimulation (condenser shocks). A, 2 shocks, 1-second interval. Note repetitive discharge on second shock. B, 2-second burst at 7 per second. C, burst of 1.1-second duration at 18 per second. D, burst of  $3\frac{1}{2}$ -second duration at 20 per second. E, 1-second burst at 44 per second. Time scale, 1 per second.

### Luminescence in Animals with Central Nervous Systems

As illustrative of nervous regulation in animals with a differentiated central nervous system, I shall refer to two groups with which I have had personal experience. These will provide examples of intracellular luminescence at invertebrate and vertebrate levels.

#### Polynoids

In polynoid or scale worms, flashes are produced by the dorsal elytra under appropriate stimulation. The photocytes involved are innervated by fibers which emerge from a ganglion in the center of the elytrum. The normal response to a single shock is a series of



flashes, with intervals of 0.1–1 second, and flashing continues for a minute or more (Fig. 5). Each flash is very brief, having a latent period of some 19 milliseconds, and lasting 83 milliseconds; and fusion and summation attend rapid stimulation, above 8 per second (Bonhomme, 1942; Nicol, 1953).

Repetitive flashing is regulated by the peripheral elytral ganglion and fails to occur in its absence, a single shock then inducing a single flash. Of particular interest is the fact that peripheral facilitation is well displayed. The first few responses show a progressive augmentation of maximal intensity, and the increment in intensity between successive impulses is inversely related to the stimulation interval (Fig. 5B). There is thus evidence for accumulation and decay of facilitator (Nicol, 1954a).

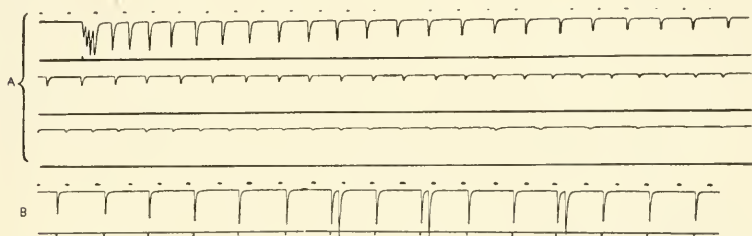


FIG. 5. Luminescent responses from single elytra of *Acholoe astericola* (polynoid worm). A, prolonged flashing following a single electrical stimulus. This is a continuous record. B, single flashes induced by repetitive stimulation at a slow rate (42 per minute). Note facilitation. Time scale of these records, 1 per second (Nicol, 1953, 1954).

Luminescence is normally evoked reflexly by tactile stimulation: afferent impulses enter the central nervous system and are relayed peripherally in efferent pathways to the elytra. Excitation is also transmitted up and down the nerve cord and causes adjacent segments to flash. Another notable feature of the response is that it is provoked by autotomy of the scales. The elythrophere possesses a visible breaking plane, and when a scale is cast off under tactile stimulation, it begins to flash rhythmically. This is occasioned either by the discharge of impulses from the central nervous system at the same time as autotomy is induced, or by rupture and stimulation of nerve fibers during autotomy. In any event the resultant impulses excite the elytral ganglion, provoking rhythmic discharge and flashing.

## Teleosts

From the viewpoint of the evolution of the vertebrate nervous system, one of the most intriguing aspects of photogeny in fishes is the character of nervous regulation. Some pelagic teleosts, notably myctophids, can emit very brief flashes with short latent periods. Receptors are tactile and visual. The innervation of the photophores has been worked out in three forms, viz., *Argyrolepecus*, *Cyclothone*, and *Lampanyctus* (Handrick, 1901; Gierse, 1904; Ray, 1950). None of these workers traced the neural pathways involved, but from the pattern of distribution of efferent nerves I hazarded the suggestion that they might be autonomic (Nicol, 1952a).

Earlier work on the midshipman *Porichthys* showed that the photophores could be excited by electrical stimulation of the whole fish, and by injecting adrenaline or pituitrin. Since nerve fibers to the photophores were little in evidence, it was concluded that regulation of luminescence was hormonal (Greene, 1899; Greene and Greene, 1924). I find that electrical stimulation of the spinal cord (condenser shocks) causes all the photophores to luminesce, both those of the head and trunk. Transection of the cord, anterior to the electrodes, and ligaturing the heart fail to prevent the response.

The latent period of the response under electrical stimulation is only 7–10 seconds, a period far too short to be explained by hormonal excitation. The circulation time of fish is not known, but is likely to exceed 10 seconds. Moreover, when adrenaline is injected directly into the heart (0.1 mg in a 1.5-kg fish), it takes 2 minutes for the luminescent response to appear, and this at a concentration which can be considered highly potent. It is apparent that the luminescent responses of *Porichthys* and certain other teleosts appear far too quickly to be governed primarily by endocrine mechanisms.

These observations can be explained most easily by postulating that the photophores of *Porichthys* are innervated by the sympathetic nervous system. The ability to respond in the absence of circulation shows that the endocrine system is not essential to the response. Teleosts generally possess well-defined sympathetic trunks which connect with cranial nerves, including the facial (Young, 1931). The latter provide avenues for peripheral distribution of sympathetic fibers

in the head. In *Lampanyctus*, and other teleosts, all the photophores of the head are innervated by the facialis, and those of the trunk are innervated by rami of spinal nerves.

The sympathetic system is the only longitudinal efferent pathway, traversing the entire length of the fish, that is available after transection of the cord. By assuming that photophore innervation in *Porichthys* is similar to that worked out for other teleosts, we can form a picture of the photophores of the head receiving their sympathetic fibers via the facialis, and those of the trunk receiving sympathetic fibers which traverse recurrent gray rami and spinal nerves in each segment. The fact that the photophores are excited by adrenaline is compatible with sympathetic innervation, since this is a normal chemical transmitter of sympathetic fibers, and the photophore nerves may well be, nay probably are, adrenergic in character. Since suprarenal tissue is well represented in teleosts, the possibility remains that the secretion of adrenaline into the bloodstream is a contributory factor in a prolonged response.

### Regulation of Light Emission by Screening Devices

Screening devices for regulating light emission make use of muscular movement, chromatophore movement, or a combination of both. Two fishes, *Photoblepharon* and *Anomalops* possess a bacterial luminescent organ under each eye. That of *Photoblepharon* is provided with a fold of opaque tissue which can be raised over the light organ. The organ of *Anomalops*, on the other hand, can be rotated on a hinge at its anterodorsal end, so as to turn the light surface inward. Steche (1909) has described a pair of antagonistic muscles which serve these organs, but their innervation is unknown. Similar devices are also known in other teleosts and in cephalopods (Harvey, 1922, 1952).

Another screening device in teleosts involves movement of chromatophore pigments. Certain fishes such as *Acropoma* and *Leiognathus* possess internal glands containing luminous bacteria. The light from these organs shines through the translucent body wall, and the presence of chromatophores in tissues overlying the photogenic organs suggests that they may be involved in regulating light emission. These organs shine continuously, and the chromatophores, by concentration

or dispersion, intensify or weaken emission without occluding it entirely. The light of *Secutor* and *Gazza* is said to increase suddenly in brilliance when the fish are strongly stimulated (Haneda, 1950). There is still some uncertainty about the mode of regulation obtaining in teleost chromatophores, but it is at least certain that pigment concentration is achieved by sympathetic stimulation and administration of adrenaline, and that the chromatophores are innervated by the sympathetic nervous system (Parker, 1943, 1948). Tactile stimulation is known to cause chromatophore concentration and blanching in some fish and is possibly a significant factor in transient intensification of light emission in fish with bacterial light organs (Osborn, 1939).

Chromatophores, overlying light organs, also control emission in cephalopods. In these animals, however, the chromatophores are actuated by muscle fibers, which expand the pigment cell when they contract, and allow it to contract when they relax. The appearance of light on concentration of chromatophores and occlusion on expansion of chromatophores have been described in *Watasenia scintillans*. As Harvey (1952) has pointed out, direct control of light production may also be involved in these screened photophores.

### Modes of Direct Control of Photogeny

The basic problem in control is turning the light on and off. Moreover, luminescence is a response with certain characteristics and parameters which, theoretically, can be varied in five ways, viz., in quality (spectral emission), intensity, duration, spatial distribution, and frequency or repetition. In actuality, we find that control of one or more of these variables is exercised by animals.

### Inhibition

Perhaps the simplest and most direct form of control consists of inhibition of luminescence by illumination. This can be exercised in either of two ways: by direct inhibition of photogenic material, or by reflex inhibition in the sensori-neural system. It is probably significant that nearly all instances of direct inhibition of photogenic material or photocytes are confined to the Protozoa, Coelenterates, and Ctenophores, animals either lacking a nervous system or provided with a nerve net. It is variously reported for *Gonyaulax* (personal observa-

tion of F. Haxo), and *Noctiluca* (contradictory observations; see Harvey, 1952), pennatulids, possibly *Pelagia* and generally in ctenophores (Peters, 1905; Moore, 1924; Heymans and Moore, 1924; Harvey, 1952).

Primary inhibition of this kind effects conservation of photogenic material in daylight, and the material is reserved for use in darkness. Such a mechanism presents functional simplicity and advantages in organisms with restricted and common modes of regulation of diverse

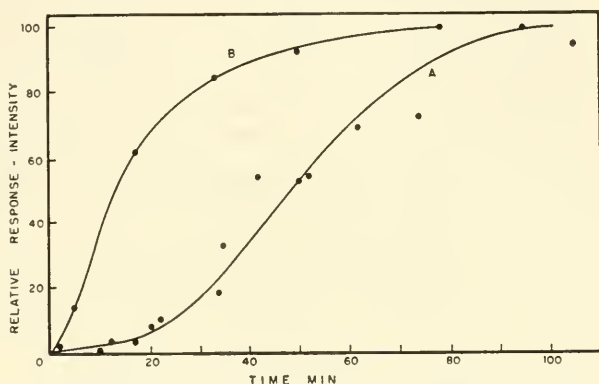


FIG. 6. Course of recovery of luminescence in *Renilla* when transferred from light to darkness. Ordinate: intensity of response evoked by a burst of shocks. Abscissa, time in minutes after transferring animal from light to darkness.

responses. In *Renilla*, for example, we find that luminescence attains full intensity only after 1 to 2 hours sojourn in the dark (Fig. 6). Now, tactile stimulation in this animal evokes both movement (contraction) and luminescence, and it is highly probable that all these responses are controlled by the same nerve net. Nevertheless, the dependence of luminescence on previous dark exposure ensures that the luminescent response is reserved for times of darkness, even though photocytes and muscle fibers are reached by efferent impulses from tactile receptors at all times.

Reflex inhibition of luminescence by daylight through photosensitive receptors might be expected, but no instances appear to have been recorded. Certainly, many animals, with differentiated central nervous systems, will luminesce when reflexly stimulated in daylight, e.g., polynoids, *Chaetopterus*, *Amphiura*. There are some suggestive



observations for the enteropneust *Ptychodera bahamensis*, in which luminescence is elicited with difficulty by tactile stimulation after exposure to light. The photocytes themselves appear to be unaffected by illumination since they still respond normally to electrical stimulation (Crozier, 1917).

Another form of inhibition is theoretically possible for intracellular luminescence. One can picture an animal in which photogeny is normally the active phase at the effector level and is continuous in the absence of external control. Inhibitory fibers would then act by suppressing light emission. Analogies are at hand in the transitory inhibition of continuous ciliary activity, as in veliger larvae (Carter, 1926). In fireflies, Buck (1948) has described four types of light emission, ranging from continuous glow to quick flash, and suggests that these may represent progressively more effective modes of control. Fireflies showing the continuous glow are unable to control the response; intermittent glow and flash represent various levels of control. It is still uncertain how control is achieved, but it can be argued that it involves some form of inhibition, either by direct nervous influence, or by restriction of oxygen supply. Records of induced luminescence in *Lampyrus noctiluca* are shown in Fig. 7.

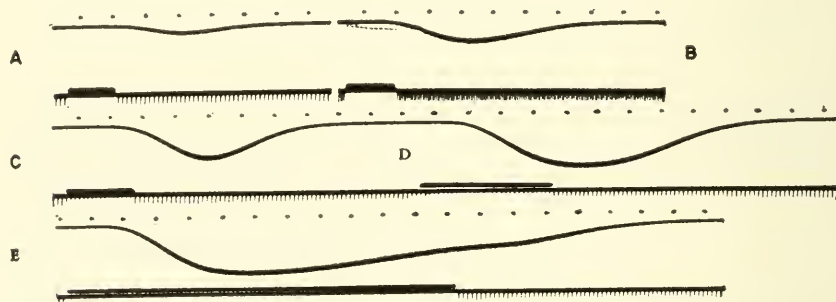


FIG. 7. Luminescent responses of the European firefly *Lampyrus noctiluca* to electrical stimulation. Electrodes were positioned over the ventral body wall and stimuli consisted of condenser shocks. A, short burst at a low frequency (2-second burst of 11 pulses at 7 per second). B, 2-second burst at 18 per second (28 pulses). C, short burst (16 pulses), and D, a long burst (28 pulses), at 8 per second. E, long burst of 90 pulses at 8 per second. Heavy horizontal line on bottom trace indicates position and duration of stimulation. Time scale above, 1 per second.



## Excitation

Let us consider now positive excitation of luminescent organs and the ways in which the response can be modulated and controlled. The luminescent response is often a triggered response, i.e., a single impulse will set it into operation. This is the case in *Pelagia*, *Beroë*, *Polynoe*, *Chaetopterus*, and *Pyrosoma*, to mention some established

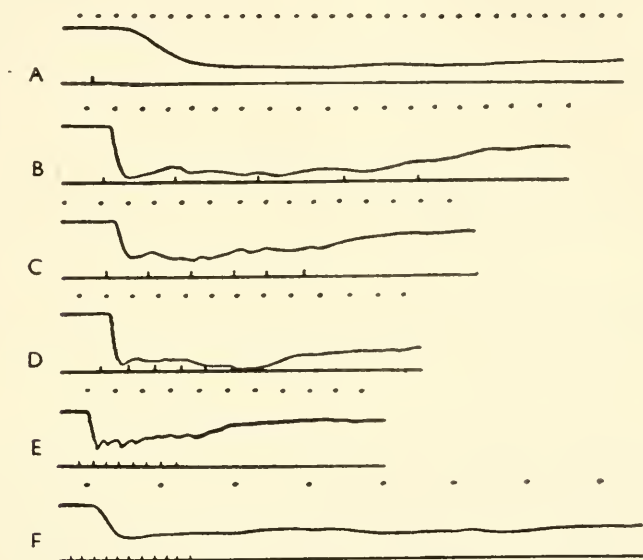


FIG. 8. Luminescent responses of *Chaetopterus variopedatus*, a polychaete which discharges a luminous secretion. A, response to a single electrical shock. Time scale 1 per second. B-F, responses to bursts of electrical shocks at frequencies shown. Time scale, 1 per 10 second (Nicol, 1952).

examples (Figs. 2, 4, 5, 8, 9). In the nerve net of pennatulids (*Renilla*, *Cavernularia*) on the contrary, facilitation is operative in the initiation of the response, as manifested by the several shocks necessary to produce the first flash (Fig. 1) (Harvey, 1917; Buck, 1953, 1955). There is also the observation that in *Porichthys*, a burst of impulses is necessary to bring out the response.

### Intensity of Response

The intensity of response is influenced in two ways, either or both of which may be operative in the same animal: these pertain to summation and facilitation. Consider first *Chaetopterus*, an animal which discharges luminous matter (extracellular luminescence). The glow from this secretion lasts for as long as five minutes (Fig. 8). Now, facilitation is not operative peripherally in the luminescent responses of this animal, but with the protracted mode of response, summation occurs readily at low frequencies of stimulation (above 2

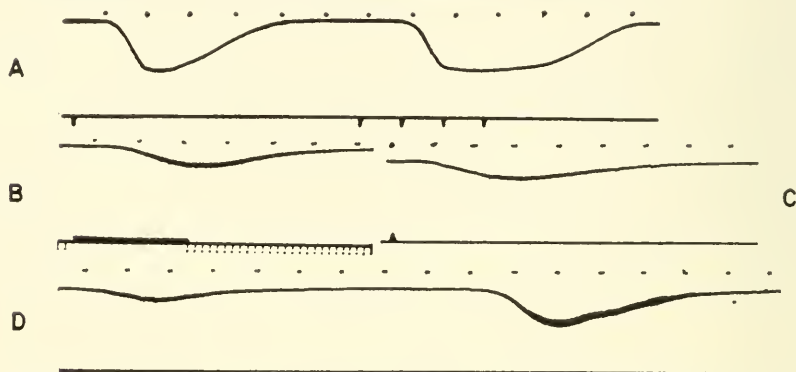


FIG. 9. Luminescent responses of *Pyrosoma*, a colonial pelagic tunicate. A, electrical stimulation with 1 and 4 condenser shocks (the latter at 1 per second). B, 2-second burst of electrical stimuli at 5 per second. C, response to flash of light (pocket torch); approximate position of stimulation added to lower line. D, responses to weak (left) and stronger (right) tactile stimulation (vibration). Amplification of A ten times that in B-D. Time scale 1 per second.

per minute). Increased intensity of response results, therefore, from several or many stimuli, compared with one, and from short compared with long interval between impulses (Nicol, 1952b,c, 1954b).

Similar response characteristics are encountered in *Pyrosoma* (Fig. 9). In this animal the response lasts some 4 seconds after a single electrical stimulus. Facilitation is not operative, at least not at slow frequencies of 1 per second. Prolonged stimulation at high frequencies, however, results in augmentation of light intensity as the result of summation of individual responses. Similarly, a strong mechanical

stimulus produces a brighter glow than a weak one, as the result of stronger tactile excitation producing more nerve impulses. The mode of luminescence in *Pyrosoma* is not known with certainty, and the problem is still obscured by Pierantoni's claim that light is due to intracellular symbiotic bacteria.

In many animals, where the response is intracellular, luminescence takes the form of a short flash or series of flashes, and the intensity is often governed by facilitation, e.g., *Renilla*, *Beroë*, *Polynöe*. In

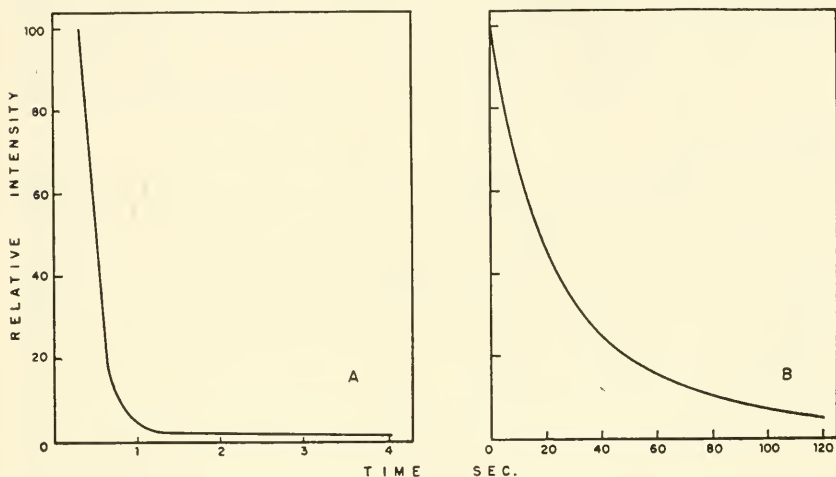


FIG. 10. Facilitation-decay curves for *Renilla* (A) and *Polynöe* (B). In A, *Renilla*, the specimen was stimulated with paired shocks, separated by intervals ranging from 0.25 to 4 seconds. The first response appeared on the second shock, and ordinates represent the intensity of response at different stimulation intervals. B, *Polynöe*, shows increment of second over first response to paired shocks, separated by intervals ranging from 1 to 120 seconds (from Nicol, 1954).

*Renilla* and polynoids at least, facilitation occurs peripherally. It manifests itself by progressive increase in intensity of successive flashes and depends in some way upon arrival of successive impulses at the photocytes. It is generally assumed that augmentation of response is owing to a banking up of facilitator faster than it can be removed (Fig. 10). This effect is observed under normal modes of stimulation (tactile), as well as in response to electrical shocks, and, therefore, is probably a factor regulating the intensity of response to environmental agencies.

### Response duration

Here we are concerned with two distinct phenomena, viz., duration of a single flash or glow and total duration of repetitive flashing.

In those animals in which a response or glow is elicited by each pulse, lengthening of response duration is achieved by prolonged stimulation. Thus, in *Chaetopterus*, which secretes a luminous material, continued electrical stimulation, at frequencies high enough to produce fusion of separate responses, also results in a longer, more durable glow (Fig. 8). This is a consequence both of summation in a contractile mechanism, responsible for expressing secretion, and of an accumulation of photogenic material at a rate faster than it can be consumed.

The second method concerns regulation of repetitive flashing. Continued flashing once stimulation has ceased, or the evocation of many flashes by a single stimulus, has been observed in *Renilla*, *Beroë*, and generally in luminescent polynoids (Figs. 1, 4, and 5). A strong tactile stimulus, or protracted electrical stimulation often sends *Renilla* into a hyperexcitatory state in which it continues to flash repeatedly, sometimes for long periods. This state of maintained flashing is an external manifestation of rhythmic discharge in the nerve net, and it would appear that nerve cells in *Renilla* can be charged up to high levels of excitability, when they pass into a rhythmic oscillatory state, expressing itself in periodic discharge across the nerve net. In polynoids repetitive flashing is the characteristic mode of response, and even a single electrical stimulus sets the elytrum flashing. In these animals the response is regulated by a peripheral elytral ganglion, which passes into some form of oscillatory condition when excited and discharges with great regularity for many seconds. In these instances, which I have mentioned, the response can be greatly prolonged beyond the original stimulus by maintained excitatory states engendered in the nervous system, be it nerve net or ganglion (Nicol, 1953, 1954a; Buck, 1953, 1955).

### Spatial Distribution

In pennatulids, possessing a nerve net, each luminescent response sweeps over the entire surface of the animal. There are many instances

known, however, of animals in which the luminescent response can be quite localized, e.g., in teleosts where separate photophores or groups of photophores light up independently of others. Among lower animals I cite *Chactopterus*, in which the response can be caused to invade an increasingly greater number of segments by increasing frequency and number of stimuli. Moreover, the response spreads with greater facility posteriorly than anteriorly. Here we have instances in which the locus of response is controlled by the central nervous system, by functional polarization, facilitation, and possibly by central representation of peripheral fields in higher forms (teleosts and cephalopods).

### Control of Spectral Emission

There are a number of animals possessing several differently colored photophores, capable of emitting light of different colors, e.g., the beetle *Phrixothrix* and the squid *Thaumatolampas*. In some of these animals the differently colored photophores can respond independently of each other, thus providing light of different colors according to the stimulus. The possibility also exists, moreover, in certain animals in which the light is controlled by chromatophores, that these organelles may influence the color of the light emitted, according to their condition and pigment characteristics.

### Conclusions

Regulation of luminescence must be related to neuro-effector control in general, and in fact, involves control of four different mechanisms, viz., glandular secretion, muscular contraction, chromatophore movement, and intracellular photogeny. Most recent work on neuro-effector, and in particular, neuromuscular, control is being carried out at the molecular level, in an attempt to discover the details of transmitter action. Regulation at the interface between nerve fibers and light organs is liable to display great variation in different systems and in different animals. Accepting differences in transmitter action, we may, in the last analysis, discover much uniformity in patterns of energy release effecting the photogenic response.

In those animals which hitherto have been studied, overall regulation of luminescence is achieved by transmission of an excitatory state, either across the surface of the cell (protozoa, eggs), or through

nervous pathways (metazoans). There are no unequivocal instances of hormonal regulation known, although this mode of control is not necessarily improbable. The interesting condition in the colonial tunicate, *Pyrosoma*, still awaits examination. In this animal a luminous wave progresses over the zoöids making up the colony. Panceri (1873) found a common system of muscles extending between the zoöids and suggested that associated nerve fibers might serve for the transmission of excitation affecting the photocytes. Since illumination evokes luminescence, it is also possible that the luminescent wave may be propagated by this means.

In the study of any physiological process, one is assisted greatly in the design of suitable experiments by an appreciation of its role in the economy of the animal. It is indeed a curious fact that although certain aspects of luminescence have advanced greatly, notably the biochemistry of the process, we rarely have any clear appreciation of the significance of the luminescent response to the animal. As an example of how knowledge of the purpose of the response can influence the design of experiments, I refer to the studies of Buck (1948) on the role of luminescence in the mating responses of the American firefly *Photinus pyralis*.

Studies now available invite speculation on another aspect of luminescence, namely restricted modes of luminescent control. It is probable that neuro-effector control was established initially for muscular systems and extended secondarily to other effectors, including luminescent organs. It is not surprising, therefore, to find certain modes of regulation common to muscular and luminescent systems. Not only has luminescence arisen independently in several different groups of animals, but each of the several forms of luminescence has also appeared independently on several occasions, e.g., discharge of luminous secretion in worms, balanoglossids and many other forms; complex photophores in squid, euphausiids and teleosts; opaque, screening lids in squids and teleosts; and different processes of chromatophore regulation in the same two groups. Within a few limited forms of structural patterns, combination and variation of detail provide multiplicity of response mechanisms. In the words of Sir Thomas Browne: "Studious observations may discover more analogies



in the orderly book of nature, and cannot escape the elegance of her hand in other correspondencies." (*Garden of Cyprus*).

#### ACKNOWLEDGMENTS

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## DISCUSSION

**Dr. Harvey:** For many years I have felt that records of the luminous response of a unicellular organism such as *Noctiluca* would be of great interest and value for comparison with single muscle fiber contractions or nerve cell potential changes. A graduate student, Mr. J. J. Chang, and I endeavored to obtain and culture *Noctiluca* for this purpose but without success, and therefore turned to the ctenophore, *Mnemiopsis leidyi*, common at the Marine Biological Laboratory, Woods Hole. Luminescence appears from a group of cells within the radial canals. Last summer Mr. Chang made an exhaustive study of this luminous response to mechanical stimulation and electrical stimuli of various frequencies. The luminescence intensity was recorded by photomultiplier tube, amplifier, cathode-ray oscillograph, and camera. The relationship between light emission and strength and duration of stimulus, repetitive stimuli, and fatigue at different temperatures has been particularly studied.

The most interesting result is the almost exact parallel between a luminous response and a muscle contraction. At 21° to 23° C with single square-wave stimulation and a small piece of luminous tissue, a single flash appears having a latent period that varies considerably, a half-rise time of 35, a maximum-peak time of 60, a half-decay time of 48, and a 0.9-decay time of 114 milliseconds. Lowering the temperature prolongs the time course of single flashes, especially the decay phase. Raising the temperature has the opposite effect. Light intensity increases as strength of stimulus increases, and repetitive stimuli elicit responses similar to summation of twitches, treppe, incomplete and complete tetanus of muscle. Fatigue appears soon and is a marked characteristic of the luminescent response. Repetitive flashes after a single stimulus have been observed in large pieces of tissue. The conduction rate of a luminous excitation along the canals averages 14 cm/sec. Bursts of action potentials, simultaneous with the luminescent responses, appear. An abstract of the work has appeared in *Science* (119, 581, 1954) and the complete paper in the *Journal of Cellular and Comparative Physiology*. Thus another organism in which the time relations of a luminous response are now well known can be added to those enumerated by Dr. Nicol.

**Dr. Mason:** It is well known that the superficial colors of many organisms are under hormonal control. In one type of control of this

pigmentation the pigment granules are either aggregated or dispersed within the cell. Is there any evidence that the photogenic granules within photocytes can be similarly aggregated or dispersed under extracellular influences?

**Dr. Nicol:** There is no evidence at present available for hormonal control of movement of photogenic granules. There is, however, the interesting observation of the Greenes (1924) to the effect that injection of pituitrin (extract of posterior lobe of the pituitary) induces luminescence in the midshipman (*Porichthys*). It is possible, although conjectural, that intermedin, the melanophore-expanding hormone, was the effective constituent in the extract. So far as I can determine, the Greenes made only a single test and did not continue the work.

As I recall, Dr. Haneda has made an interesting observation concerning certain species of squid that possess a luminescent mantle organ partially bounded by the ink sac (e.g., *Euprymna*). It seems that movement of ink in the ink sac could be controlled in such a way as to blacken or expose the surface of the light organ and thus regulate light emission.





## Some Reflections on the Control of Bioluminescence

JOHN BUCK

National Institute of Arthritis and Metabolic Diseases, National Institutes  
of Health, Bethesda, Maryland

From the information and data at hand it is evident that one cannot necessarily extrapolate from one luminescent system to another or from the behavior of one luminous organism to that of another. Nevertheless, the work reviewed by Dr. Nicol does point to certain tentative generalizations. For one thing, the simpler organisms such as bacteria, protozoa, coelenterates, ctenophores, and polychaetes seem either to luminesce continuously or to light up only in response to external stimuli, and it is only in groups with rather well-developed nervous systems that photogeny becomes subject to the precise sort of regulation so well exemplified in the mating signals of fireflies. Secondly, it is clear that the ability to emit sharply delimited flashes of light does not depend on a highly developed nervous system but on intracellular light production. Thus, for example, in *Chaetopterus* and *Cypridina*, animals with well-developed nervous systems, light production is extracellular and the luminosity decays slowly, whereas the primitively organized *Noctiluca*, *Renilla*, and *Mnemiopsis* produce flashes not inferior in temporal control to those of many fireflies. Extracellular luminescences are characterized, in addition, by rapid "fatigue" due to exhaustion of the luminous excretion, whereas intracellular photogeny may sometimes persist through many hundred successive flashes.

The main emphasis of Dr. Nicol's paper is on the properties of the nervous systems of various luminous organisms (and in this he presents a strong case for photogeny being as accurate and critical an

index of neural activity as is muscular contraction) rather than in the actual linking of the nerve impulse to the control of light production. However, I was happy to see that his records confirm the observation that facilitation occurs at the neurophotocyte junction in *Renilla* (Buck, 1953), because this suggests a parallel between photogenic control and events occurring at the motor end plate. In this connection, and in apparent opposition to Parker's (1919) generalization that effectors have preceded neural regulation in evolution, Dr. Nicol makes the interesting suggestion that the photogenic system may have been able to hook into an already existent and widespread control mechanism, the neuromuscular system. This may make less puzzling at least the existence of diverse but highly developed photogenic control systems throughout the animal kingdom, if not the apparently haphazard development of the photogenic capacity itself (Harvey, 1952).

The presence of two effector systems, muscles and photocytes, dependent on a single conduction system, has necessitated a notable degree of coordination even in so simple an animal as *Renilla*, in which light production and zooid retraction may occur together or independently. At the same time, as Dr. Nicol has suggested, the inhibition of luminescence by light, as seen particularly in the more primitive luminous organisms, may have utility in sparing luminous substrate for the hours of darkness. In view of the teleological attractiveness of this speculation it would be of interest to know whether the photoinhibition involves, as in *Mnemiopsis* (Moore, 1924), the control system (and in this I suggest the possibility of direct action on the nerves as well as via light-sensitive receptors), or also, or instead, photochemical destruction of the photogenic substrate as suggested by Dr. Harvey's (1921) experiments with *Bolina*. In the latter instance it would be desirable to show that reversing the photoinactivation, or photodegradation, is less demanding, energetically speaking, than the synthetic replacement of substrate simply allowed to "burn" irreversibly.

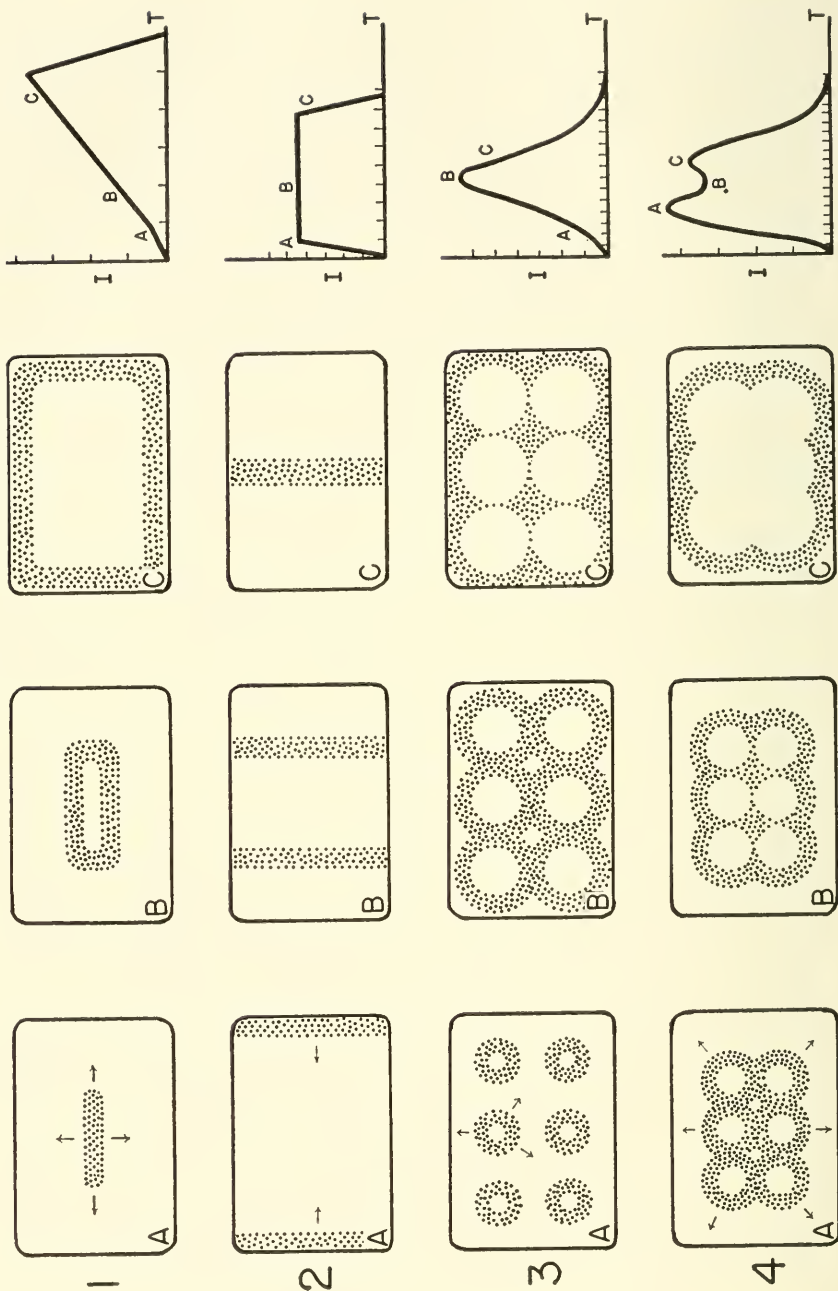
Dr. Nicol's figure for the response of *Pelagia* seems to indicate that duration of flash declines as intensity falls off in consecutive flashes. This would be contrary to the findings of Brown and King (1931) on fireflies and to what would be expected in luminous extracts, where

duration would not change with increasing total light (total luciferin) as long as the enzyme-substrate ratio was unchanged. Actually, of course, the shortening of the *Pelagia* responses is an artifact of amplification and would disappear if the intensities were equalized, but it nevertheless justifies some discussion of the general relation between the kinetics of light emission by the organism as a whole and the kinetics of light production in the underlying photochemical system. The discussion will perhaps be useful also in reference to the striking similarity in form (though not in absolute rate), pointed out by Dr. McElroy, between the "pseudoflash" given by anaerobic firefly extracts upon admission of oxygen and the normal flash of the intact firefly.

Although twenty years ago it was still possible to think of the actual light-emitting reaction as involving primarily two reactants, luciferin and luciferase, and although the decay portion of the duration-intensity curve of some firefly flashes is logarithmic, Brown and King (1931) and Snell (1932) clearly recognized the difficulty of relating the control of luminescence directly to the underlying chemistry. At our more sophisticated level of knowledge today, with many enzyme cycles involved, there is much more difficulty in fixing upon the rate-limiting reaction except in highly purified, *in vitro* systems.

In extracellular luminescence, as in *Chaetopterus*, obvious kinetic complications are caused by extraneous factors such as secretion delay and mixing delay (Nicol, 1952). However, even considering intracellular photogeny and making the simplifying assumptions that the response is triggered by a single nerve impulse and that the photogenic cell is a unit (with all quanta being emitted simultaneously and with the accretion and decay phases clearly dependent on defined reactions), a formidable degree of kinetic indeterminism may enter purely at the biological level of organization as soon as the response of intact animals is considered. This is well illustrated in *Renilla* where the response is sufficiently leisurely for one to see that several ranks of siphonozooid clusters (each cluster being itself composed of several individual siphonozooids and each siphonozooid of several cells) are involved at a given instant in each luminous wave. One must sternly eschew, therefore, the temptation to guess that the beautifully reproducible and quite possibly logarithmic decay of

PLATE I



## PLATE I

Schematized diagrams of the spread of luminescence (stippled areas) in light organs stimulated in various hypothetical ways and the corresponding intensity-duration curves which would be obtained by recording from the whole organ.\* The three stages of spread of excitation (A, B, C) are indicated at the corresponding points on the graphs which form the last member of each series. The concentric spread of luminescence can be thought of as reflecting either a sort of nerve-net propagation of excitation from the initiating areas through successive ranks of effector units (as in *Renilla*) or as the pattern of direct effector innervation, the apparently progressive spread from each focus indicating conduction delay from the underlying stimulation center (as would be more likely in the firefly). It has been assumed that only a single stimulus is given to each effector unit (repetitive stimulation would of course alter the curve forms drastically) and that a given area of organ lights only once. If the decay of luminescence were long in comparison with the rate of spread of excitation, i.e., if the stimulated regions stayed lit at least until the whole organ was excited, all the patterns of excitation would give the same general type of intensity-duration curve.

FIG. 1. Luminescence spread in an organ initially stimulated centrally. A possible instance of this sort of propagation is the circular prothoracic photophore of the elaterid beetle *Pyrophorus* in which the flash is so slow that the spread of luminescence is easily observed. In this organ, Heinemann (1886) observed that the light appeared first at the center of the organ and spread peripherally, and died out in reverse order. (If the stimulation began peripherally, peak intensity would be gained early, giving a curve skewed to the left.)

FIG. 2. Luminescence spread in an organ in which the excited region is of constant area. The same form of intensity-duration curve is obtained if the excitation starts centrally and spreads laterally in both directions, and if there is only one excited region instead of two. An example of such propagation is seen in the long slender rachis of sea pens.

FIG. 3. Luminescence spread in an organ with multiple excitation points. This type of propagation, which is the most likely type in lampyrid fireflies, gives a roughly symmetrical intensity-duration curve which is quite nonspecific.

FIG. 4. Multifocal luminescence spread giving a bimodal type of intensity-duration curve reminiscent of that of the female of the firefly *Photuris pennsylvanica* (Brown and King, 1931). The initial phase of this curve is equivalent to Fig. 3A, but the subsequent spread of luminescence differs in that the excitation points are relatively closer together in relation to the total area of the organ so that after the initial peak is reached (at A), the die-away of luminescence at the 7 points of impingement of the 6 centers of spread exceeds, for a time (4B), the increase in luminous area due to peripheral spread of excitation. Later the peripheral area increase becomes dominant (4C), followed by a decay essentially similar to that in Fig. 3C.

\* Thanks are due Dr. Margaret Keister for making the necessary calculations and executing the figures.



*Renilla* luminescence, as seen in Dr. Nicol's records, directly reflects the underlying chemical kinetics. There is indeed one possible deduction which can be made about *Renilla*, though not from Dr. Nicol's present records (which integrate the light from the animal as a whole), which is that the time-intensity curve for the individual siphonozoid cluster must be skewed. This is shown by the fact, noted by Parker (1920), that the leading edge of the wave (first rank of clusters) is the brightest.

The firefly flash is even more difficult to analyze because its brilliance and short duration prevent visual study of the course of excitation through the luminous tissue, and no recordings have been made of the time relations between light emission from different regions of the organ in the same flash. Since the organ itself occupies two abdominal segments in the males of most common American fireflies, asynchrony between the two segments is a distinct possibility, although the early firefly experiments of Brücke (1881), leniently considered, indicate that the innervation could conceivably be so arranged that the two segments are stimulated simultaneously somewhat as in the unified response of the squid mantle (Young, 1938). Even so, one would be dealing with from 6000 to 15,000 effectors, depending on whether one considers the "cylinder" or the photocyte to be the smallest individually controlled unit (Buck, 1948), so that it is likely *a fortiori* that the time-intensity curve for the whole organ represents an integration of many separate asynchronous events rather than the simultaneous firing of all the photocytes in the organ. The strong resemblance of the time-intensity curves of some firefly flashes to the normal distribution curve may be significant in this connection, although I understand from Dr. Hastings that a symmetrical flash is the exception, rather than the rule. However, a gaussian type of curve might reflect the distribution of any one of several factors affecting the control of luminescence, for example the distribution of responsiveness among the individual effectors to a linear change in stimulation frequency (action potentials in nerve). Similarly, plateau-shaped, bimodal or skewed time-intensity curves might result rather simply from the mere architectonics of innervation in the light organ when the recording is made from the animal as a whole (Figs. 1-4),



or, assuming  $O_2$  to be the controlling factor, the tracheation of the organ (cf. Heinemann, 1886).

Dr. Nicol's passing reference to the possibility of inhibiting luminescence by limiting oxygen invites amplification. The case has in one sense been strengthened by the recent evidence that oxygen actually participates in the reaction in which the activated molecule emits the light—formerly it was possible to imagine a more subordinate role analogous to that in hexose resynthesis in muscle. As an essential reactant in light production in almost all organisms, oxygen is of course always a potential trigger at the chemical level, but the possibility of its actually ever normally being limiting is strongly reduced by two circumstances. First, no organism, except possibly the firefly (see below), seems to have any anatomical arrangement even conceivably capable of rapidly affecting the oxygen supply to the luminous tissues. Second, in all systems thus far investigated (which includes the firefly) luminescence persists at oxygen partial pressures far lower than will support any significant amount of respiration. Dark periods, which are often of great length, would thus involve almost complete tissue anaerobiosis.

Since oxygen control has often been postulated for the firefly, it may be worth while to examine the evidence thought to favor this view. Oxygen control has been strongly espoused by Snell (1932) and Alexander (1943), but in a critical review of the work (Buck, 1948) I concluded that no evidence had been presented which could not be interpreted as an indirect effect via the nervous system, rather than a direct limitation of oxygen in the photochemical reaction. Aside from the obvious dependence of light production on the presence of oxygen, therefore, we have only the characteristically profuse tracheation of the light organ, which may of course be concerned with supplying some oxidative precursor or restoration reaction rather than with control, and the circumstantial but close correlation between the ability of certain species to produce sharp flashes and the presence in those species (only) of "tracheal end cells" at the junction of the supply tracheae with the fine tracheal capillaries (tracheoles) which penetrate the photogenic tissue. From the standpoint of pure plumbing the end cells are indeed strategically situated to retard

gaseous diffusion into the photogenic tissue. However, the volume of the tracheoles is negligible compared with that of the tissues, and the final effect of oxygen limitation would in any case be seen in the cytoplasm and must involve diffusion of oxygen in solution. Hence, some reason must be given why dissolved oxygen will not diffuse into the photogenic tissue from the surrounding and presumably well-oxygenated blood, and from contiguous tissues. Furthermore, the slowness of diffusion of dissolved gases, particularly at the very low  $pO_2$  and small gradients at which limitation would occur during the decay phase of luminescence, raises serious doubt that the observed time constants for the flash could be achieved. End-cell control was examined exhaustively in my 1948 review, and the conclusion was reached that control at the enzymatic level, probably via nervous triggering, was much more likely—a notable piece of clairvoyance, in view of Dr. McElroy's recent ingenious acetylcholine proposal.

The effects of different oxygen tensions on the luminescence of intact fireflies (Snell, 1932; Alexander, 1943; Buck, 1948) bring up another facet of the control problem. Normally, captive fireflies do not luminesce visibly while at rest, but if the ambient  $pO_2$  is gradually reduced to about 4 mm, an "hypoxic glow" (not a flash) develops, which persists steadily for a long time. If the  $pO_2$  is suddenly increased, the luminescence suddenly increases to a high level and then declines to zero ("pseudoflash"). If the  $pO_2$  is decreased still further, the hypoxic glow dies out. These phenomena have been interpreted in terms of a control mechanism which is inactivated at low  $pO_2$ , allowing oxygen to diffuse into the photogenic tissue unchecked but producing only a dim light because of the low  $pO_2$ . The pseudoflash, on this interpretation, would represent a period of bright luminescence due to the increased  $pO_2$  and terminated quickly by the recovery of the control mechanism. (The die-out with decrease in  $pO_2$  below 4 mm would of course signify a straight oxygen limitation.)

Snell (1932) interpreted the control mechanism directly in terms of the end cell, and I (1948) suggested the possibility of the pseudoflash being due to burn-off of luciferin accumulating during hypoxia as in the bacterial "flash" (according to Dr. McElroy's present firefly scheme this would represent oxidation of accumulated "active inter-

mediate"). The Snell hypothesis is subject to the objections inherent in any hypothesis of control by oxygen limitation. The hypothesis that formation of active intermediate is rate-limiting in normal control has the attractive feature that the rate could be affected either by oxygen limitation (the hypoxic glow-pseudoflash phenomenon) or, in normally oxygenated animals, by making some other reactant limiting such as acetate, as proposed in Dr. McElroy's nerve-action control suggestion. There is, however, a possible obstacle in that I have not been able to demonstrate a relation between length of hypoxia and magnitude of pseudoflash. Possibly this obstacle can be resolved with additional work.

The end-cell control hypothesis, though seriously defective in some respects, has in it the germ of a possibly important concept. As I pointed out in 1948, the mechanism which controls the hypoxic glow-pseudoflash phenomenon ought to be one which requires energy expenditure, that is, the animal has to do work to keep itself dark. This would accord well both with the effects of hypoxia and with the fact that fireflies develop a long-lasting glow during anesthesia and after death. Such an "active dark" mechanism need not be and ought not to be restricted to oxygen limitation. Although it is perhaps premature to propose specific neuroeffector or chemical control mechanisms in either fireflies or other organisms, there is at least the suggestive analogy of skeletal muscle, in which work is done to maintain the tissue normally in the relaxed state, and the well-established presence, in some Arthropods, of inhibitory nerves.

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## DISCUSSION

**Dr. Davenport:** To the interesting discussions presented by Dr. Nicol and by Dr. Buck I should like to add a few remarks in regard to an aspect of bioluminescence beyond that of the physiological mechanisms of control.

Greatest emphasis has been placed during the conference on the biochemistry of luminescence, for the obvious reason that the phenomenon has paramount importance as a tool of the investigator of the basic metabolism of cells. Some emphasis has been placed on mechanisms of control of luminescence, which in multicellular organisms must of necessity be investigated over the years in each differing luminous species. Some emphasis, largely descriptive but of great interest, has been placed on the natural history of luminous organisms. But the subject of the experimental analysis of behavior in luminescent organisms has barely been touched upon.


It is obvious that a careful analysis of the behavior of luminous forms should give us considerable information concerning the importance of luminescence in their evolution. Although the question of the adaptive significance of luminescence in particular continuously arises, practically no investigations resembling the interesting ones of Dr. Buck on Lampyrids have been undertaken.

As an example of a case of luminescence which has been used, without comparative studies, to bolster important evolutionary theory we have that of the fungivoroid Diptera. Goldschmidt has used the New Zealand species to support the theory that by necessity there must have been sudden great evolutionary changes in morphology, physiology, and behavior for the New Zealand form to have been brought to its present level. It now appears as a result of Dr. Haneda's observations that there are luminous members of this single dipterous family as far separated as New Zealand, North Carolina, and Japan. Such discontinuous distribution in closely related luminous forms indicates that the habit of luminescence in these Diptera, although it is not the same in each species, may be of as great geological antiquity as that of the Lampyrids.

Unquestionably, studies in comparative behavior in related luminous forms should give us a clearer picture of their course of evolution; let us hope that in the future more investigations will be devoted to this interesting facet of the whole subject of bioluminescence.







## Luminous Organisms of Japan and the Far East

Y. HANEDA

Tokyo Jikeikai Medical College, Tokyo, and  
Yokosuka Museum, Yokosuka, Japan

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My investigation of luminous organisms started in 1934, with special interest in the problem of luminous symbiosis, between luminous bacteria and fish or squid. From 1937 to 1942 I had several opportunities to visit Micronesia, Tropical Asia, and New Guinea as a member of the staff of the Palao Tropical Biological Station and was able to collect luminous organisms and observe their ecology. From 1942 to 1945 I was stationed in the Raffles Museum, Singapore. During my stay in Singapore I was able to visit the Malay Peninsula and the East Indies. During these trips I collected and observed luminous fishes, luminous fungi, fireflies, and other luminous organisms. Unfortunately, most of my specimens, memoranda, and manuscripts were lost at the end of World War II while traveling in Middle Sumatra. Finally in 1946 I returned to Japan from Singapore, and in 1948 I again started to study deep sea luminous organisms of Suruga Bay, Japan.

From 1951 to 1952 studies were made of luminous organisms of Hachijo Island, located 157 miles south of Tokyo, while I was a member of the Committee on Oceanographic and Biological Research of Hachijo Island. Since 1953 I have continued the work on luminous species of the Pacific coast of Japan, as a member of the Committee on Oceanographic and Biological Research for Marine Resources, sponsored by the Japanese National Commission for UNESCO. The present paper contains some of the results of my observations on the

various remarkable and interesting luminous organisms of Japan and the Far East.

### Bacteria

In Japan very few taxonomic reports appear on luminous bacteria, but there are many observations on these organisms. Studies on morphology, immune reactions, cultivation, relation to pH and temperature, effect of salt, action of drugs, antibiotics, and others have been made by many workers, namely, Imamura (1904), Yasaki (1926), Ninomiya (1924), Majima (1931), Kishitani (1933), Takase (1938, 1939), Nakamura (1939, 1940, 1942a,b), Haga (1942), Yasaki and Kimura (1946), Yasaki and Kobayashi (1946), Kozukue (1952a, b), Odawara (1953a,b,c), and Shibata (1953a,b).

#### Symbiotic Luminous Bacteria

Studies on symbiotic luminous bacteria have been made by Yasaki (1928, 1929), Kishitani (1928a,b,c, 1930, 1932), Yasaki and Haneda (1935a,b), and Haneda (1938a,b, 1940, 1941, 1950, 1951).

My own work has been chiefly concerned with fishes of the Acropomatidae, Leiognathidae, Gadidae, and Macrouridae, from which the following conclusions can be drawn. Two species of luminous bacteria exist in two species of *Acropoma*, namely, *Acropoma japonicum* Günther and *Acropoma hanedai* Matsubara; one group of luminous bacteria from fish of the Leiognathidae, and one group of luminous bacteria from fishes of the Gadidae and Macrouridae are all different and constitute new species. The details of the bacteriological work will be presented elsewhere.

#### Parasitic Luminous Bacteria

Studies on parasitic luminous bacteria were made by Yasaki (1927), Majima (1931), and Haneda (1939).

Nonluminous crustacea and insects frequently become luminous when infected with luminous bacteria. Infection of fresh water shrimp has been investigated by Yasaki. The infected shrimp live only a few hours but they are brilliantly luminous. The luminous bacteria were isolated and called *Microspira phosphoreum*. Majima (1931), through his detailed study, later reidentified it as *Vibrio yasakii*.

Nonluminous Isopoda sometimes become luminous from infection by luminous bacteria. A specimen of *Megaligia*, Japanese name, *Funamushi*, among numerous nonluminous individuals, was observed at Tomioka Beach near Yokohama in the autumn of 1933. Again in 1939 in Palao, I observed a wood louse, Japanese name, *Warajimushi*, and a millipede infected by luminous bacteria moving on the ground behind the coral storehouse of Palao Tropical Station. The rather strong light from the wood louse was emitted from the whole body and lasted five days. The normally nonluminous millipede *Trigoniulus rugosus* is common in the Caroline and Marshall Islands of Micronesia, but occasionally luminous bacteria can be cultured from these animals.

### Saprophytic Luminous Bacteria

There are many reports of luminous bacteria cultivated from marine fishes of Japan, and especially since World War II much progress has been made by Dr. Yasaki and his students in studies of luminous bacteria. Kozukue (1952b,c), Shibata (1953a,b,c), and Odawara (1953c) cultivated luminous bacteria from the digestive organs of some marine fishes, squid, and crab.

The only observation on luminous bacteria from beef was made by Molisch (1926) at the city of Sendai, Japan. Several years before 1940 I tested the cultivation of luminous bacteria from beef, pork, and chicken in Japan. The beef, pork, and chicken, purchased from butcher shops in several cities, were cut into small pieces with sterilized knives, put into a dish, and sterilized salt water was poured on them. After 10 to 24 hours I observed these in the dark. Although there was some luminosity developed in experiments conducted during other seasons, in the winter season 65% of the beef, 46% of the pork, and 24% of the chicken became luminous. Strangely enough the luminous bacteria grew only on those purchased in the city of Ohgaki, Gifu Pref. I cultivated these luminous bacteria in culture media containing 0.5% salt and found that the bacteria emitted light strongly.

Important research, using luminous bacteria as tests for antibiotics (Adawara, 1953a,b); for study of spectral distribution (Akaba, 1938; Takase, 1940; Haneda, Takase, and Kumagai, 1940); for observation with the fluoromicroscope (Shibata, 1953d), and for mutant investi-

gation (Iizima and Kikkawa, unpublished) have been made, but space does not allow a description of the results.

During World War II, luminous bacteria were grown for illumination in blackouts, and recently an extract of luminous bacteria called "Florads" was made for sale by Dr. Takino (1953) of the Dainippon Zoki Institute for Medical Research, Osaka, Japan. Florads costs 500 yen per box of 5 ampules, each containing 2 cc for injection. According to Dr. Takino, Florads has an anti-allergic and neurotropic action and acts effectively upon rheumatism of the joints and spontaneous gangrene without deleterious effects.

### Fungi

It is a most remarkable fact that many species of luminous fungi appear at night in the rainy season in the forests or jungles of the tropics. The decayed wood that had grown luminous fungi was collected and brought back to Japan from tropical countries, where the fungi continued to glow in my laboratory in Tokyo during the summer season and I was able to observe in detail their ecology, the intensity of light, and color of their light.

Eight new species of these luminous fungi from Micronesia and North Borneo were identified by the late Dr. S. Kawamura. The results of my observation of the luminosity were published by me (1939), and a taxonomical report was made by Kawamura (1940) in Japanese. Five more new species were also identified by Kawamura, but he died in 1943 before he was able to publish his final taxonomical report. *Pleurotus lunaillustris* and *Mycena bambusa*, named by Kawamura, are widely distributed in the tropics. In Singapore Dr. E. J. H. Corner and I collaborated in our collection and observations of luminous fungi and some of our findings were published by Dr. Corner (1950).

#### Luminous fungi of Japan

On the Pacific coast of South Japan and adjacent islands, some genera of luminous fungi are found which are common in Micronesia, East Indies, and the Malay Peninsula.

In Japan the following four luminous species are well known: *Lampteromyces japonicus* (Kawamura) Singer, *Armillaria mellea*

(Vahl) Karsten, *Dictyopanus pusillus* (Lev), Singer, and *Mycena cyanophos* from Bonin Island. Recently Dr. Kobayashi (1951) reported the following five luminous species from Miyazaki Pref. of South Kyushu, Japan: *Poromyцена Hanedai* Kobayashi, *Dictyopanus folucolus* Kobayashi, *Mycena pseudostylobates* Kobayashi, *Mycena daishogunensis* Kobayashi, and *Mycena* sp.

*Lampteromyces japonicus* is a large luminous fungus which grows on the dead trunks of beech trees in autumn. It is famous for the Japanese name *Tsukiyo-dake*, meaning "moon night mushroom." It is a poisonous species and closely resembles the nonluminous and edible species *Pleurotus ostreatus*. According to Dr. S. Kawamura (1915), the luminescence comes only from the lamellae of the fruit-body, and not from the spores. However, according to my observations, spores fallen upon moist blotting paper are slightly luminous. *Polyporus Hanedai* Kawamura (1940) is the synonym of *Poromyцена Hanedai*. I found it in a jungle near Tawao, North Borneo, in March 1938. In August, 1953, Mr. Okuyama collected this fungus which had grown on decayed trunks of mulberry trees at Hachijo Island. Three other luminous species were found by us on Hachijo Island: *Mycena chlorophos*, *Dictyopanus gloeocyst* Corner, and *Mycena lux-coeli* Corner. The last mentioned species, illustrated in Fig. 1, is very common.

### Tropical Luminous Fungi

Recently Dr. Corner has identified specimens of luminous fungi from the tropics and from Hachijo Island. According to him, seven *Mycena*, two *Poromyцена*, two *Dictyopanus* and one *Pleurotus* were identified as *Mycena chlorophos* (B. & C.) (Palao, 1937; Celebes, 1943; Hachijo Island, 1952), *Mycena rorida* sp. nov. (Singapore, 1944), *Mycena pruinoviscida* sp. nov. var. *rabaulensis* var. nov. (Rabaul, 1942), *Mycena lux-coeli* sp. nov. (Hachijo Island, 1951), *Mycena sublucens* sp. nov. (Amboina, 1942), *Mycena noctilucens* Kawam (Yap, 1937), *Mycena illuminans* Henn (Singapore, 1944), *Poromyцена Hanedai* Kobayashi (Borneo, 1938; Rabaul, 1942; Miyazaki, 1951; Hachijo Island, 1952), *Poromyцена manipularis* sp. nov. (Ponape, 1940), *Dictyopanus luminescens* sp. nov. (Singapore, 1944), *Dictyopanus gloeocyst* sp. nov. (Hachijo Island, 1951), illustrated in



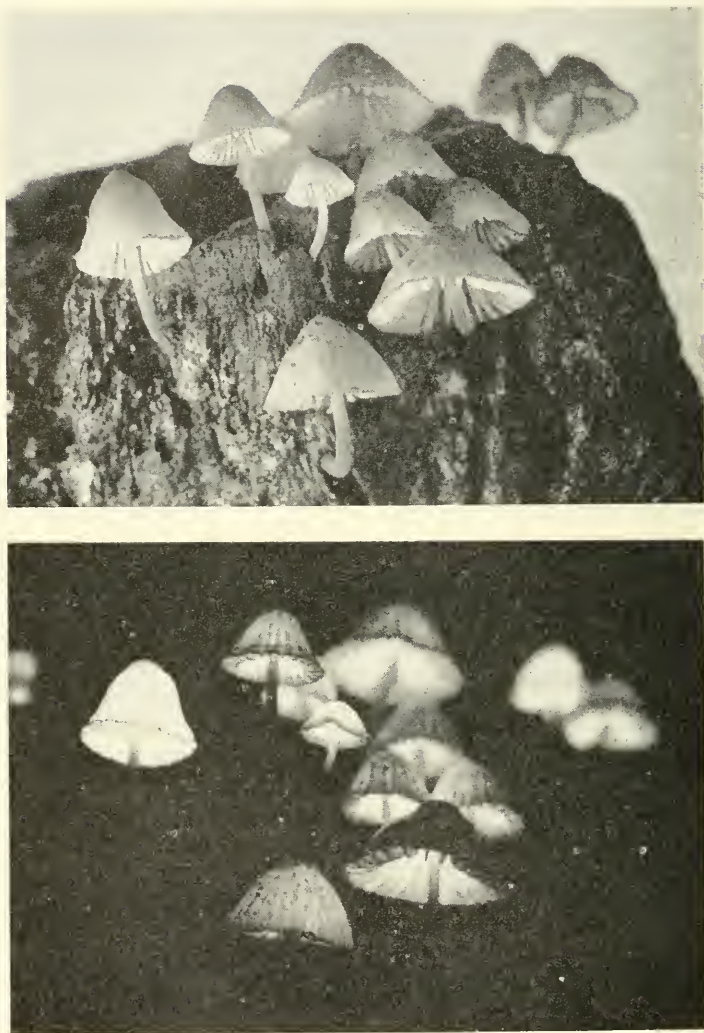


FIG. 1. The luminous fungus, *Mycena lux-coeli*, from Hachijo Island, Japan, photographed by daylight (top) and by its own light (bottom).

Fig. 2, and *Pleurotus noctilucens* Lev, (Palao, 1937; Yap, Saipan, Rota, Truk, Ponape, 1940; North Borneo, 1938; Manukwari, New Guinea, 1942; Singapore, 1943; Java, 1944). *Mycena bambusa* Kawamura is a synonym of *Mycena chlorophos*, and *Pleurotus lunaillustris* is a synonym of *Pleurotus noctilucens*.



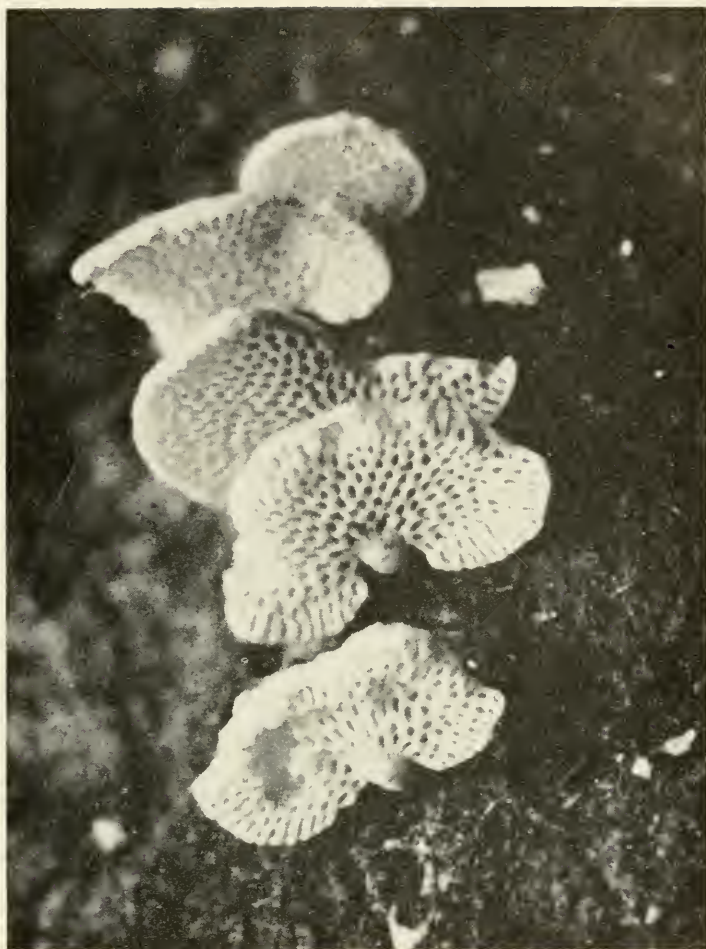


FIG. 2. *Dictyopanus gloeocyst*, a luminous fungus from Hachijo Island, Japan.

Among these the most interesting fungi are *Mycena pruinoviscida* var. *rabaulensis* from Rabaul and *Mycena rorida* from Singapore. Both species are remarkable among many luminous fungi because only the fresh damp spores which have fallen out of the fruitbody are luminous. The spores which fall on a dry place are not luminous, but they become luminous when water is dropped on them.

From Formosa Dr. K. Kominami (1930) reported a luminous species, *Mycena photogena* (Japanese name *Hotaru-Dake*, meaning

"firefly fungus"). According to him, the fruitbody of this fungus is green in color, and the color of the light is blue. Lamellae and spores are luminous.

In India Dr. Bose (1926, 1930) reported that leaves, stalks, grass roots, and living roots from the forests of Bengal were luminous. As the result of pure cultivation from these luminous leaves, he decided that the light of leaves might be due to various species of *Mycena*.

I have cultivated the luminous mycelium from spores of various luminous fungi and observed and measured the intensity of light, color of light, spectral distribution, and the relation to temperature. This work will be published in a separate paper.

#### Other Miscellaneous Luminous Fungi

Besides the above-mentioned luminous fungi, I collected on my expeditions to tropical countries the following luminous species identified by Dr. Kawamura: *Mycena phosphora*, *Mycena microillumina*, and *Marasmius phosphorus* from Palao, *Mycena vapensis* from Yap Island, and *Mycena citrinella* var. *illumina* from Ponape Island. Unfortunately, these specimens were lost during World War II in Tokyo and therefore have not been reidentified by Dr. Corner.

The luminous fungi hitherto known all belong to Hymenomycetinae, which comprise the families of Agaricaceae and some Polyporaceae. However, I collected in January, 1940, a minute luminous fungus which Dr. Kawamura identified as belonging to the Nidulariineae, shown in Fig. 3. These minute fungi were found on the bark of *Rhizophora mucronata* in a mangrove zone in Markyok village of Palao. Under the microscope they can be seen to emit a light, bluish in color.

#### Dinoflagellata

In Japan taxonomical studies of Dinoflagellata have been made by such workers as Nishikawa (1901), Okamura and Nishikawa (1904), Kofoed (1931), and T. H. Abe (1927, 1936, 1940). Many luminous species are known, but in the above reports the luminosity was not recorded.

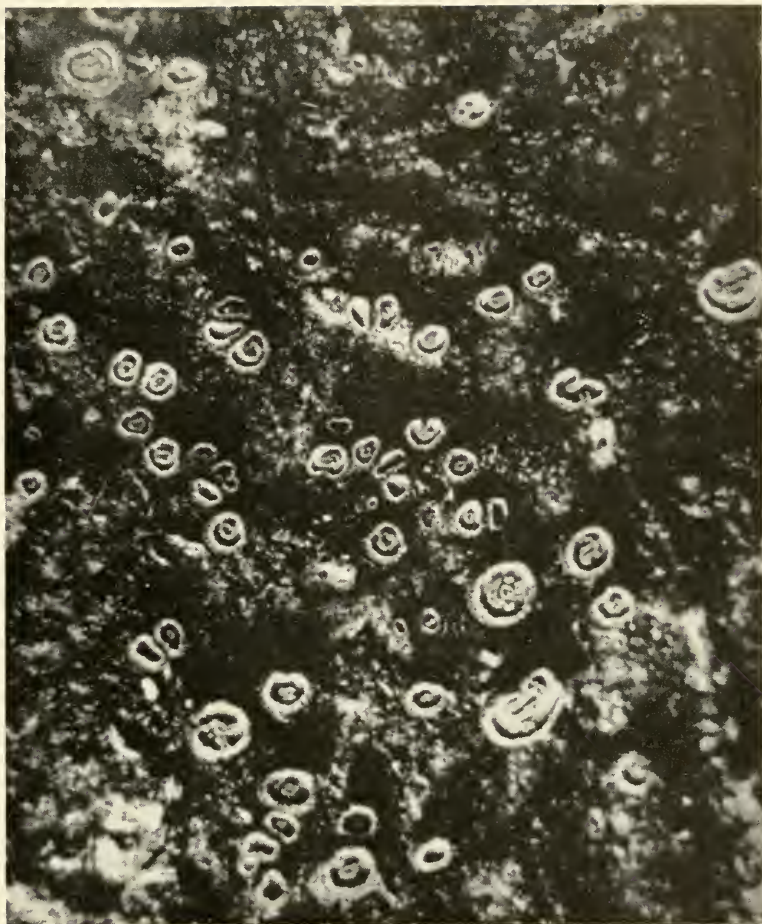


FIG. 3. Minute luminous fungi of the family Nidulariineae from Palao, Micronesia ( $\times 54$ ).

### *Noctiluca miliaris*

*Noctiluca* is one of the most remarkable luminous organisms, abundant in Japanese waters throughout the year. Especially from April to June large quantities of *Noctiluca* appear, and masses of them floating on the sea are sometimes blown to shore by the wind. A mass of *Noctiluca* can be several centimeters in thickness, making the sea about it gelatinous and coloring the water a reddish brick. Japanese

fishermen call it "Akashio," meaning "red stream." Many fish are at times killed by such a stream, and the masses are strongly luminous at night.

*Noctiluca* does not occur in the Pacific coral islands, such as Palao, Saipan, Truk, Yap, Ponape, and Marshall Islands, but appears abundantly near the waters of continental islands, such as New Guinea, Sumatra, Borneo, Java, Celebes, and also along the coast of the Malay Peninsula and Indo-China. However, I have collected it in the sea 300 miles north of the Celebes, where it developed along the coast and was carried far distances by an ocean current.

*Noctiluca* from Japan is pale pink in color, but the tropical species from the East Indies and New Guinea are green. The green color is due to symbionts, which Weill (1929) has called *Chloroflagellates*, while Hada (private communication) considers them *Chlamydomonas*. Ostroumoff (1924) reported that green *Noctiluca* is not luminous, but I have observed brilliantly luminescent green *Noctiluca* on the north coast of West New Guinea and other islands of the East Indies, and also in Sandakan Bay of North Borneo. The luminosity of Sandakan Bay was the most beautiful.

Dr. Ueno (1937) found *Noctiluca* and the luminous species, *Ceratium fusus*, and also *Peridinium* sp. in the swamp, Mokoto-Numa of Hokkaido, Japan. This swamp was connected with the sea, the upper surface being fresh water and the bottom sea water. According to him, *Noctilucae* were floating 2 to 3 meters deep at the surface. There were 44,000 per liter, and the color, due to green *Chlamydomonas*, was green as in all tropical species. Unfortunately the luminosity of these *Noctilucae* was not observed.

### Macroplankton

Reports on luminosity of the macroplankton such as jellyfish, *Pyrosoma*, and *Salpa* are almost lacking in Japan. The only reference found was one on eggs of a Ctenophore, by Dr. Yo. K. Okada (1926).

T. Komai has reported ctenophores (1918-21) in the neighborhood of the Misaki Marine Biological Station and also cubomedusae (1938) on the northern Pacific coast of Japan. Uchida (1928, 1929) described Japanese Hydromedusae around the coast of Japan, and T. Kawamura (1915) reported Siphonophores in the vicinity of Misaki. While



probably luminous, the light of these specimens was not actually observed.

In my own observations, luminous jellyfish appear abundantly along the coast of Japan, especially from March to June. About the end of April, 1953, I collected many luminous *Pelagia* in a flying fish net near Hachijo Island, and in May of the same year observed luminous *Pelagia* in great numbers in Yokosuka harbor and along the west coast of Izu Peninsula.

In the flying fish nets at Hachijo Island, many luminous Ctenophora such as *Beroë* and *Cestum* are caught. In June, 1953, along the coast of Namerikawa, Toyama Bay, numerous *Beroë* are often mixed with luminous squid, *Watasenia scintillans*, the whole net becoming brilliantly luminous. Several species of Siphonophora, such as *Praya*, *Diphyes*, and *Abyla*, which were caught in a plankton net at Aigae Bay, Hachijo Island, were also observed emitting light.

In May, 1953, I collected *Aequorea* sp. by trawl net in Suruga Bay. This is a rather large Medusa, 200 mm in diameter, which emits a flash of light on irritation.

In September, 1943, in the sea around the Thousand Islands, coral islands off Jakarta, Java, species of Ctenophora which emitted a flash of light when irritated were found.

### Observations by Undersea Observation Chamber

Twice I have had the opportunity of observing luminous organisms in the sea at night while riding in an undersea observation chamber, called "*Kuroshio*" (Inoue, Sasaki, and Oaki, 1952, 1953).

The observation chamber was 3.15 meters in height, 1.48 meters in diameter, and weighed from 4440 to 5220 kg, with a buoyancy of from 4192 to 4297 kg. The chamber had a main observation window (150 mm diameter) with controllable reflector, 3 auxiliary observation windows (100 mm diameter), telephone, teletalk, oxygen feeder, CO<sub>2</sub> absorbing unit, projector lamps and miscellaneous gages. The *Kuroshio* observation chamber was hung down along the outside of the hull of the mother ship by means of a steel wire pulley on the end of a derrick. The lowering and raising was done by an electric motor winch, usually at a speed of about 20 meters per minute.

In all, 130 diving observations were made in 1952 along the Japan

coast. The following summer observations were continued off the coast of Otaru, Hokkaido.

My first experience was in December, 1952, in the sea off Ito, Izu Peninsula, and the second was in August, 1953, in the sea off Otaru, Hokkaido. In both descents, conducted at night, I saw the luminescence of *Beroë* and *Cestum* under natural conditions and at a depth of about 20 to 50 meters. I can never forget the beautiful luminous appearance of the transparent, long, bandlike *Cestum* through the window glass of the chamber, as the animal emitted a very beautiful flash of light. At the same time I saw numberless luminous Copepoda running across the window like a snowstorm.

Below the surface, suspended materials like snowflakes could be seen with the naked eye, wherever the beam of the projector light fell. According to the laboratory experiments of Drs. Suzuki and Kato of Hokkaido University, these suspended flakes are assumed to be aggregates of disintegrating bodies of plankton organisms. These unidentified masses have been named "Marine snow" or "Sea snow" or "Plankton snow." One night in the summer of 1953, in the sea off Otaru, Hokkaido, I observed that some of these suspended flakes were luminous like the ctenophore *Beroë*. They were suspended in the water and, coming gradually to the window glass, they were crushed to minute luminous spots and then faded out. My own opinion is that luminous bacteria grow on the suspended flakes, causing them to be luminous.

### *Pyrosoma* and *Salpa*

There is one report on a possible luminosity of *Salpa* in Japan. According to Tokioka (1937) *Cyclosalpa pinnata* var. *polae* (Sigl), 18 to 22 mm in length, appearing at Seto, Wakayama Pref., has one luminous stripe, but he never saw light from these individuals in the dark. *Pyrosoma* and *Salpa* are common in Japanese waters. About the middle of April, great numbers occur in the waters of Hachijo Island.

These *Pyrosomae* have been identified as *Pyrosoma atlanticum* Péron and two species of *Salpa* as *Thetys vagina* (Tilesius) and *Pegea confoederata* (Forskal) by Dr. T. Tokioka. Another small species of *Pyrosoma*, which was caught in a trawl net in Suruga Bay was identified as *Pyrosoma verticillatum* Neuman.



The two above-mentioned species of *Pyrosoma* are brilliantly luminous when stimulated. The species of *Salpa* are not luminous under natural conditions. They emit light only on strong stimulation, such as cutting or tearing the body. As the luminous regions were small and the periods of luminosity very short, I could not determine the position of the luminous organs.

## Annelida

### Polychaeta

There are many species of luminous Polychaeta in Japan. However, no reference to their luminosity has appeared except in *Chaetopterus variopedatus* and *Mesochaetopterus japonicus* Fujiwara (1935). *Chaetopterus variopedatus*, which appears to be a species of worldwide distribution, is found on the sandy bottom of Japanese waters and also along the west coast of Korea. Fujiwara (1935) described somewhat different light regions occurring in the *Mesochaetopterus japonicus* of western Japan.

During my stay in Palao, I collected some species of luminous Polychaeta. Among them *Onuphis* sp. and *Stylarioides parmatus* Grube (1878), both identified by Mr. K. Takahashi, are the most interesting species. *Onuphis* sp., belonging to the family Eunicidae, lives in coral in the neighborhood of the Palao Tropical Biological Station, Corror Island, Palao. This Polychaete, a beautiful pink in color, with brown stripes, is 234 mm long, 5 mm wide, and has 204 segments. The pairs of luminous spots are arranged from the first setigerous segment to the last on the abdominal lateral margin of each segment. The animal emits a bluish green light when stimulated, but it does not discharge a luminous slime. It is closely related to the species of *Onuphis investigatoris* Fauvel from the Arabian Sea, the luminosity of which is unknown.

A single specimen of *Stylarioides parmatus* was collected from the coral reef of the Corror Island, Palao. It lives in a tube 20 mm long and 2 mm in diameter, which is attached to a dead shell. Its cephalic plate becomes luminous when irritated, emitting a yellowish green light. No luminous slime is discharged. This species occurs in the Philippines, Ceylon, Madras, Madagascar, and New Zealand, but its luminosity has never been reported.

In April, 1953, I collected several pelagic species of luminous *Tomopteris* with a plankton net in Aigae Bay, Hachijo Island.

At the pearl cultivating station of Usa, Kochi Pref., I recently collected luminous *Thelepus* sp., the external appearance of which is similar to that of *Polycirrus*. These organisms live in the living shell of pearl oysters and the tentacles become luminous when stimulated.

### Oligochaeta

In Japan there appear two species of luminous earthworms. One is terrestrial (*Microscolex phosphoreus*) and the other is a sea earthworm (*Pondodrilus matsushimensis*). *Microscolex phosphoreus* is worldwide in distribution. In Japan it was first found in 1934 at Oiso, Kanagawa Pref., and later it was collected by many persons in every part of Japan. This worm is small, 40 mm in length and 1–1.5 mm in diameter, pale pink in color, and translucent. It is not luminous under normal conditions but after strong stimulation it discharges luminous mucus from mouth and anus. In November, 1939, Mr. Kuroki, a teacher of the Fukuoka Middle School in Kushu, observed many luminous earthworms crawling on the paved road near Fukuoka. On that cold rainy night they were trod under foot by pedestrians and crushed by bicycles and cars, and the soles of shoes, the tires of bicycles and cars became brilliantly luminous. I also collected some in my private garden at Zushi, Kanagawa Pref., in November, 1948. As these species are small and are not luminous under normal conditions, they are not known by most of the Japanese people.

The sea earthworm is widely distributed along the coast of Japan. The body, which is pale pink and translucent, is about 100 mm in length and has 100 to 105 segments. The luminosity of this worm was discovered by the late Dr. Kanda and myself when we saw them in the wet sand at the tidal line near Yokohama. This worm is also not luminous under normal conditions, but it will discharge a yellow luminous mucus from mouth and anus on strong stimulation or injury. I have collected luminous sea earthworms at the tidal line of tropical countries of Asia. According to Dr. Obuchi, these tropical species are not the same as the Japanese species.

I have also endeavored to cultivate luminous bacteria from the luminous fluid of both species of worms and have obtained negative

results. Microscopic examination did not reveal any luminous bacteria. On blotting paper the luminosity of the luminous mucus faded out after a few minutes while the paper was drying, but dropping water on the blotting paper revived the luminosity. The color of the light of luminous mucus on the blotting paper was pale blue, which changed to yellowish when the paper was rubbed. The intensity of luminosity was also increased by rubbing.

### Mollusca Except Cephalopoda

Luminous species of the great group of Mollusca, excluding squids, are comparatively rare. Seven genera of Gastropoda, *Phyllirhoë*, *Tethys*, *Kaloplocamus*, *Plocamophorus*, *Latia*, *Dyakia*, and *Tonna*, are known to be luminous. Two genera of Pelecypoda, *Pholas* and *Rocellaria*, are also known to be luminous.

In Japan the luminous species of Gastropoda, Opisthobranchiata or sea slugs, in which the shell is absent, were studied by a number of workers. Dr. Okada and Dr. Baba observed luminous sea slugs, *Plocamophorus tilesii*, in Mutsu Bay. The mucous cells were scattered over the whole body, and the luminous slime came out of mucous cells.

Beautifully colored plates of two luminous sea slugs, namely *Plocamophorus tilesii* and *Plocamophorus imperialis*, were reproduced in the book *Opisthobranchia of Sagami Bay collected by His Majesty, the Emperor of Japan* (1949). The beautifully colored large sea slug, *Calinga ornata*, found in Japanese waters, is also known to be luminous. Recently Kato (1949a and b) reported the luminosity of the small sea slug, *Kaloplocamus ramosum*, which comes from the east coast of Japan. The animal has many luminous cells and no pores, but the luminous cells contain large granules. It emits intermittently bluish flashes of light when irritated but it discharges no secretion into the water, although other luminous sea slugs are known to do so. I have observed it in the dark at the beach of Usa Marine Biological Station of Kochi University in November, 1953, and have tested for the luciferin-luciferase and ATP reactions, with negative results. The famous transparent pelagic nudibranch *Phyllirhoë bucephala* is occasionally collected in the neighborhood of the Misaki Marine Biological Station.

Among Pelecypoda, *Pholas dactylus* was at one time thought to be the only luminous species. However, in 1939 at Palao, I found that *Rocellaria grandis*, a coral boring shell, is also luminous. This animal has a thin white shell 45 mm long, 15 mm wide, and 15 mm in height. I have collected living *Rocellaria* from the living corals *Favites virens*, *Goniastrea parvistella*, and *Porites tenuis* in Iwayama Bay of Corror Island, Palao. *Rocellaria* has a pair of luminous stripes on the mantle along the pallial lines and produces a luminous secretion which fills the mantle cavity and spouts from the siphon when irritated. The dried luminous tissue recovers its luminosity when moistened and the luciferin-luciferase reaction is positive.

Among the Gastropoda Pulmonata it was thought that *Latia neritoides* (Suter, 1890), a New Zealand fresh water limpet, was the only luminous species. It has been fully studied by Bowden (1950). However, we found a luminous snail, *Diakia striata* of the Zonitidae, in Singapore. One night in September, 1943, when Mr. Kumazawa, entomologist, was collecting luminous larvae of fireflies on the lawn of the Good Wood Park Hotel, Scot Road, Singapore, he saw a weak light from a small land snail and informed me of the possibility of luminescence in land snails. The next evening we went to the place and were astonished to observe a true luminescence in this animal.

The snail, about 10 to 15 mm in diameter, lives on grass or lawns in Singapore. The type specimen in Raffles Museum was collected from Gunung Pelai, Johore. I have also collected specimens in various regions of the Malay Peninsula and Kalimon Island of the Rhio Archipelago. The light appears inside the anterior region of the foot and cannot be seen when the animal is irritated and has withdrawn within its shell. When expanded the bluish white light passes through the translucent muscles of the head and of the foot and flickers like a firefly. The time relations of the flashing depend on the stage of development of the snail and various other conditions, such as temperature, humidity, and light. Young snails, immediately after hatching, about 1 mm in shell diameter, emit a weak luminescence over the entire foot. The light appears continuous, but on close inspection the diffused glow can be resolved into small flashes scattered over the area. As the snail grows, the flicker rate diminishes, and some full grown individuals do not luminesce at all. The normal duration of a

flash at 25° C is two to three seconds. The flashing is spontaneous; no luminescence appears on stimulation. The intensity of each flash is the same and fairly bright, visible in electric light if shaded by the hand.

The luminescence comes from luminous cells, not from luminous bacteria. The luminous organs, consisting of large luminous gland cells, lie below the mucous gland of the foot and surround its opening, but no luminous material is secreted to the outside. Hence this snail is self-luminous, and the light is intracellular.

In sections, the luminous tissue can be easily distinguished. The luminous organ of a shell 15 mm in diameter is 2 mm long and 1.5 mm wide. The luminous cells are pale green, while the mucous cells of this region are yellowish orange when stained by safranin.

### Luminescent Marine Snails

Luminous species of Gastropoda Prosobranchiata are also very rare. According to Turner (see Harvey, 1952) *Tonna galea* Linne, a marine Gastropoda, is luminescent. When this animal is moving about with its foot well extended, it emits a greenish white light.

We have found two species of luminous Gastropoda on the beach of Borawazawa, Sueyoshi Village, Hachijo Island. During ebb tide on April 23, 1953, my friend, Mr. H. Okuyama, saw some small marine snails emit light as they rolled in the sea water when he raised a stone. We were very much astonished and collected many specimens, examining them repeatedly to find out if these marine snails were really luminous, or were luminous only because of eating some luminous matter, or were infected luminous bacteria. As a result of these observations, I decided the snails are truly luminous, possessing a luminous organ on their mantles.

These luminous marine snails, shown in Fig. 4, are very small. The shell, a beautiful pale pink with brown bands, is 10.5 mm high and has a diameter of 6 mm. The scientific name of this snail is *Planaxis viratus* Smith, identified by Dr. I. Taki of the National Science Museum in Tokyo. I found another species, *Planaxis perescelida* Dall, in our collection and this also had the same luminous organ and emitted light. This species is rather more slender than the *Planaxis viratus*. Its height is 12 mm and its diameter 5 mm. The color is dark brown



with pale black or dark brown bands. Each species is widely distributed in southern Japan.

When the animal is under natural conditions on a rock with its foot well extended, no light can be seen. However, the light appears on strong stimulation. If many specimens are placed in a bottle and



FIG. 4. The luminous marine snail, *Planaxis viratus*, from Hachijo Island, Japan.

well shaken in the dark, some of them become luminous and twinkle. The light continues one or two minutes and then gradually disappears. If the body of the snail is irritated, the light reappears, and if placed in fresh water, the light continues for a longer time.

When the animal is taken out of the shell by crushing the shell and is observed under a binocular or low power microscope in the dark, the luminous region is found to be on the dorsal part of the mantle in a limited area, as shown in Fig. 5. Under the microscope numberless luminous dots can be seen appearing or disappearing. The luminescence of this animal is intracellular and the luminous organ con-



sists of many groups of luminous cells which run parallel to each other. No luminous cells are scattered over the whole body.

When the dried materials of both species were ground in a mortar and moistened with water, luminescence reappeared and then disappeared. The luciferin-luciferase reaction, tested by mixing hot water (70° C) and cold water extracts of crushed *Planaxis*, was negative. When the cold water extract is allowed to stand until the light disappears, it will not again emit light when ATP is added.

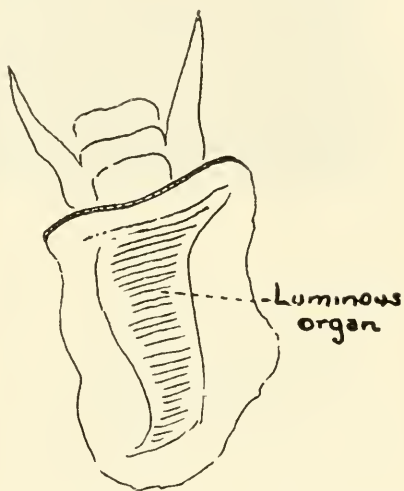


FIG. 5. A *Planaxis viratus* removed from its shell to show the luminous organ in diagram at right.

### Cephalopoda

There are many reports of luminous squid in Japan. According to Harvey (1952) luminous squid are divided into the following three groups, depending on their method of light production: (1) squid associated with luminous bacteria, (2) squid producing an abundant luminous secretion, and (3) squid with well-developed photophores and intracellular luminescence. In Japan the first and third groups have been studied by such workers as Watase (1905), Ishikawa

(1913), Harvey (1917), Shoji (1919), Y. K. Okada (1933), and Hasama (1941). However, the second group has not been investigated until recently.

Squid associated with luminous bacteria were studied by Dr. T. Kishitani. He reported that myopsid squid, such as *Loligo edulis*, *Sepiolo birostrata*, and *Euprymna morsei*, are luminous species possessing symbiotic luminous bacteria. All live in shallow water. Their luminous organs have openings to the exterior, and luminous bacteria live symbiotically in the ducts. In Singapore in 1944 I collected and observed another species of myopsid squid having luminous bacteria.

Regarding the second group, I recently caught in a trawl net at Suruga Bay a luminous squid producing an abundant luminous secretion. The scientific name of this squid is *Stoloteuthis leucoptera* Verrill, identified by Dr. Y. Okada, and is a species closely related to *Heteroteuthis*. As shown in Fig. 6, its mantle is 20 to 30 mm long, with a large white band that has been mistaken by some observers for a luminous skin organ. The round white luminous organ is situated on the ink sac and is connected to the exterior by two pores. A section of the luminous organ is similar to that of *Heteroteuthis*, which also produces a luminous secretion.

*Stoloteuthis* is not luminous when freely swimming, but, if touched, will spurt through the funnel a beautiful bright bluish secretion like that of the mollusc *Pholas*. This luminous secretion comes out of the pores of the luminous organ. I have endeavored to cultivate luminous bacteria from the luminous organ and the luminous secretion, but all the results were negative. As this luminous squid is comparatively easy to catch in trawl nets at Suruga Bay, I intend to study it in detail in the future.

The third group of squid with well-developed photophores have been studied by many workers in Japan. Among many deep sea luminous oegopsid squids, *Watasenia scintillans* is most famous and remarkable. *Watasenia scintillans* (Japanese name, *Hotaru-Ika*, meaning firefly squid) comes to the surface in Toyama Bay, on the coast of the Nippon Sea, each year during late April to mid-June, to breed. This species is caught and dried on the beach in the sun for food and is an important commercial product of Uozu and Namerikawa, towns on Toyama Bay that are the best locations for collecting.

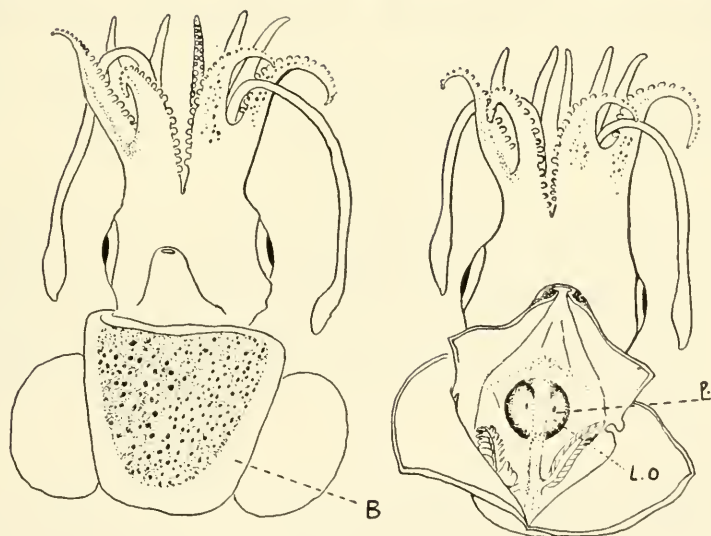
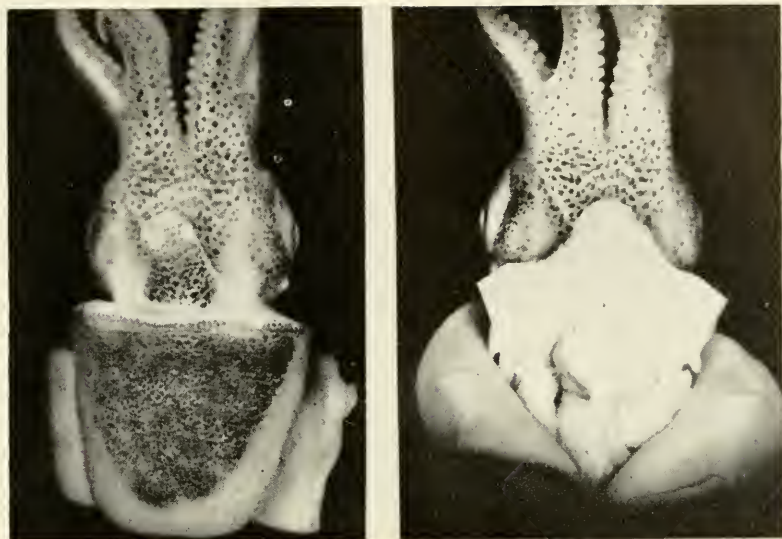


FIG. 6. Photograph (top) of luminous squid, *Stoloteuthis leucoptera*, which ejects a luminous secretion from its funnel when disturbed, with diagram of parts (bottom). Left, a dorsal view. Right, the body cavity has been opened to show the luminous organ (L. O.) with pores (p).

Other interesting deep sea luminous squids, such as *Abralia japonica*, *Chiroteuthis*, and *Calliteuthis*, are obtained in shrimp nets of trawlers in Suruga Bay. Appearing in particularly great numbers are *Chiroteuthis imperator* (Japanese name, *Yurei-Ika*, meaning ghost squid), which fishermen throw away because it has no commercial value. This squid has many well-developed luminous organs on the eyeballs, tentacles, and abdomen. The tentacles of large specimens extend 2 meters and attached to them for some length are many luminous organs, like many small lamps on one thread.

Sasaki (1915) described two new luminous oegopsids from the Bay of Sagami, namely *Meleagroteuthis separata* and *Symplectoteuthis luminosa*. The luminous organs of the former are uniform in appearance, thickly covering the ventral surface of the whole body, but are found in less number on the dorsal surface. Luminous organs are found also on the ventral and dorsal surfaces of the head and arms. The *Symplectoteuthis luminosa* were taken six miles off Misaki, at 700 fathoms in 1906. According to Sasaki, a macula is found on the ventral surface of the head. On the ventral surface of the mantle there occur a pair of longitudinal zones of the same character which run along the whole length of the mantle and are divided into three parts. He supposed the maculae and zones to be luminous organs, judging from their histological structures.

### Crustacea

Many self-luminous species appear among the orders of Crustacea in Japan. The luminous species *Cypridina*, belonging to the Ostracoda and *Heterocarpus sibogae* of the Decapoda, which secrete a luminous liquid, while *Sergestes prehensilis* of the Decapoda, with photophores, and certain freshwater luminous shrimp are the most interesting and remarkable.

#### *Cypridina*

*Cypridina hilgendorfi* (Japanese name, *Umihotaru*, meaning sea firefly), shown in Fig. 7, is abundant along the coast of Japan from July to September. A well-known crustacean, it is often used for biochemical studies. It is 3 mm long and produces a strong luminescent secretion. It can be preserved in a dried condition, and the



FIG. 7. Photo of *Cypridina hilgendorfi* from Japan.

luminescence can be restored with moisture. This *Cypridina* species lives on the sandy bottom near the shore and comes out to feed at night. There are several methods of collecting *Cypridina*. During World War II, Japanese army officers used large earthenware pots baited with fish, which they lowered to the sandy bottom at night. This method was not too satisfactory because nonluminous Crustacea and marine snails were mixed with the *Cypridina*. A simple method for mass collection is to tie fish heads to long strings and suspend them in the sea at night. *Cypridina* gather on the fish heads and may be easily caught. The living specimens are then placed on blotters in sunlight to dry, or in a heater with a low temperature. If dried while still alive, the two hinged valves become transparent, and a brilliant luminescence appears on moistening; if dried after death, the valve changes to a nontransparent white, and the luminosity is weak.

The Military Institution of Japan during World War II was planning to utilize *Cypridina* light for reading at night. I saw some samples of *Cypridina* powder in Singapore which were sent from Japan, but in tropical countries the powder putrified very rapidly when moistened, and I do not know whether it was useful or not.

*Cypridina noctiluca*, shown in Fig. 8, is a tropical species com-



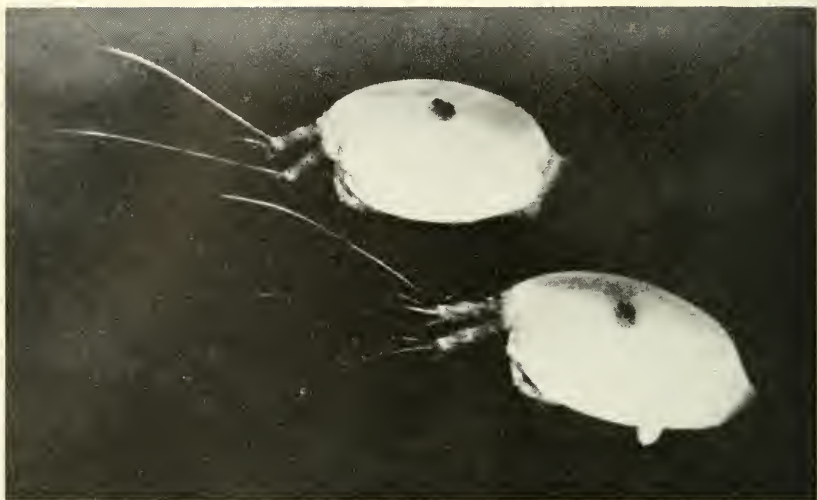


FIG. 8. Photo of *Cypridina noctiluca* from tropical Asia.

monly seen in the waters of Palao, Java, Malay, and even Hachijo Island. It is slightly smaller than *Cypridina hilgendorffii*, being 2 mm long and pyramidal in form. It is a pelagic species and can be taken only by plankton net, since the animals are not attracted to fish bait. At Hachijo Island, as in tropical seas, *Cypridina noctiluca* is abundant, but no specimens of *Cypridina hilgendorffii* appeared. The light of *Cypridina noctiluca* and that of *Cypridina hilgendorffii* are quite similar in color.

#### Luminous Shrimp

In the winter season from October to May near the towns of Yui and Kambara in Suruga Bay, Shizuoka Pref., more than 100 trawling boats go out to catch deep sea shrimp. The scientific name of these shrimp is *Sergestes prehensilis*, and the Japanese name is *Sakura-Ebi*, meaning cherry shrimp. These are dried on the beaches in sunlight and are an important commercial product of the two towns. This shrimp possesses 157 photophores scattered over the body. The trawl nets which catch the shrimp are drawn from depths of from 50 to 100 fathoms by two fishing boats, and they bring up many other kinds of luminous animals, such as other species of shrimps, luminous squid, lantern fish, hatchet fish, *Pyrosoma*, jellyfish, and



other luminous deep sea animals. These forms live mostly at middle depths and migrate upward at night. Among them the most interesting and remarkable are the shrimp and squid which project luminous clouds into the sea water. *Heterocarpus sibogae* de Man. is 5 to 6 cm long and red in color. This species, like *Heterocarpus alfonsi*, has glands at the base of the antennae. If the material is dried rapidly while living by a heater with temperature not exceeding 40–50° C, its luminosity can be recovered upon moistening. When dead material is dried, the luminosity is weak. The luciferin-luciferase reaction is positive, and the species would be useful in biochemical studies.

### Pathogenic Luminous Shrimp

The luminosity of the freshwater luminous shrimp *Xiphocaridina compressa* is much more interesting. These are found in Lake Suwa, 100 miles from the sea and 800 meters above the sea level, but are also widely distributed in freshwater in Japan.

On hot summer nights the shrimp regularly become luminous because of infection by pathogenic luminous bacteria, and the entire body, with the exception of the eye, but even tentacles and legs, will shine.

The morphological aspects of the luminous bacteria (*Microspira phosphoreum*) are similar to the Cholera Vibrio or the *Vibrio dumbar* of Germany. The bacteria are easily cultivated from the shrimp, using a 0.5% NaCl culture media. Later, in 1928, the shrimp was found in a brook in the rice fields alongside the Tone River near the town of Sawara, Chiba Pref., about 30 miles from the mouth of the estuary. Because of the rare beauty of the shrimp as they mass and luminesce on hot summer nights, their destruction is prohibited by the government.

## Myriapoda

### Luminous Centipedes

There appear to be no luminous *Myriapoda* in Japan, but small threadlike luminous centipedes (*Orphaneus brevilabiatus*), 60–60 mm long, are distributed over Micronesia, the East Indies, Malay Peninsula, and Indo-China, even Formosa and Okinawa Islands. Translu-

cent and pale brown in color, they live in the walls of native houses, in the furniture or beds made of pandanus leaves. Palao people call them *Teraí Was*, or luminous paint, and think they are ear eaters, although they are not poisonous. The animal discharges a very strong greenish luminescent slime from both sides of the body segments when irritated. If put into chloroform vapor, they secrete a luminous mucus from every segment, which is very striking. One night in 1938 at Arumizu Village, Corror Island, Palao, I saw a centipede caught in the web of a spider which was very beautiful in the luminosity of the discharged slime.

### Luminous Millipede

Records of luminous diplopods or millipedes are few. I found a self-luminous millipede in the Truk Islands of Micronesia in 1939, from which no luminous bacteria could be grown. Takakuwa (1941) gave it the name of *Spirobolellus phosphoreus*, a new species. The whole body of this animal, with the exception of head and legs, emits a weak bluish light which becomes brighter upon irritation, but it excretes no luminous mucus as in the case of luminous centipedes or earthworms. It is very common in the Truk Islands, and I saw it many times during my stay of one week there. Many of them gather at the base of coconut trees. Although the light intensity is no stronger than that of mycelium of luminous fungi, the light can be recognized from afar. Dr. Y. Kobayashi, mycologist, observed this luminous millipede on Ponape Island, but I never saw it anywhere in tropical countries except Truk.

## Insecta

### Fireflies

Regarding fireflies in Japan, Yo. K. Okada (1931) reported 33 species. However, as most of them live in Formosa, Okinawa, and Korea, we can scarcely count more than seven species in Japan proper. They are: *Luciola cruciata* Motschulsky, *Luciola lateralis* Motschulsky, *Luciola parvula* Kiesenwetter, *Pyrocoelia fumosa* Gorham, *Pyrocoelia atripennis* Lewis (Amami-Oshima Island), *Psilocladus variolosus* Olivier, and *Lucidina biplagiata* Motschulsky. Among them are *Luciola*

*cruciata* and *Luciola lateralis* species, whose larvae live as aquatic glowworms; *Luciola cruciata*, distinguished by black wings and a red cross on the head, is the largest in Japan. Its larva develops in clear streams and has a pair of small abdominal luminous organs. The firefly appears from the end of June to July, and Japanese people go to various places where it swarms to view the splendor of its flashing light. As the glowworm tends to decrease in number, many famous places for fireflies have been under protection by the government as sanctuaries. The larva of *Luciola lateralis* appears in rather dirty water in rice fields or brooks. *Luciola parvula* is a mountainous species. *Pyrocoelia fumosa* is common near Tokyo; however, only its larva is luminous. Other species seem few in number because they are not easily recognized.

In Micronesia fireflies live only in the coral Islands of Palao and Yap. The firefly *Atyphella carolinae* found in these islands is 7 mm in length and has black wings. After sunset swarms of the insects fly up for about an hour at a time and are a beautiful sight to see.

Many species of fireflies live in the East Indies, Malay Peninsula, and New Guinea, and although I collected some specimens, they were burned in Tokyo during the war. Later specimens I collected were lost in Singapore, so unfortunately I am unable to report on them.

### Synchronous Flashing of Fireflies

Although my specimens from these countries were lost, I can never forget the amazing spectacle of synchronous flashing of fireflies in New Guinea. I happened to see it in March, 1940, at the Rabaul Botanical Garden, Rabaul, New Britain. On the leaves of a big silk tree countless numbers of fireflies were alighting and flickering rhythmically, causing the whole tree to appear as if it were breathing. This species, with black wings, was 7 mm long. Its flicker is distinct, because when the light disappears, it does so instantaneously and completely. My detailed observations are as follows:

1. The silk tree was a big one, and the fireflies alighted forming three groups, one on the upper part, one on the middle part, and one on the lower part. The flicker was transmitted rhythmically, the upper group extinguishing its light first, followed by the middle

group, and last the lower group. Sometimes the rhythmical flashing was transmitted from the lower part to the upper. The flashes were repeated at the amazing speed of seventy per minute. The phenomenon continued every day for one week while I was there, lasting from sunset to dawn, notwithstanding the rain. It resembled the description reported by Smith (1935), except that he noted the phenomena occurring when the moon was half full.

2. When a strong electric light was directed on them for a few seconds the synchronous flashing became irregular. After thirty seconds new synchronisms arose from some other groups in the tree and extended over the whole tree.

3. Fireflies on the tree were male and female in equal number. This fact differs from the observations reported by Morrison (1929) and Smith (1935). I observed about 100 males and females, each in separate cages in the darkness. Only the males continued to flash synchronously. On the contrary the females showed irregular flashing.

4. Not only the luminous organs of the male and female, but the color also, differ. The difference is discernible with the naked eye. The color of the light of the males is yellow, while that of the females is bluish green. Their light looked like scatterings of yellow and bluish green powder when the tree was shaken.

5. Even after dawn, with the sun shining brightly, the fireflies remained on the leaves of the tree.

6. This species of firefly selected thin-leaved trees. Sometimes these fireflies flashed synchronously as they flew through the air.

7. Many copulating fireflies were found on the grass under the tree. At that time the males were not emitting light. In view of this observation, it seems the synchronous flashing is a behavior pattern by which the males invite the females to a group.

I do not believe there is any permanent leader in the group that acts as a continual pacemaker for the synchronous flashing. I think that when some individual or group emits light, it has a stimulating effect that causes the light to spread throughout the whole group as a wave.

The larva of this firefly is terrestrial. I collected a luminous pupa in the Botanical garden at Rabaul.

In March, 1943, I saw again this beautiful synchronous flashing at

Manukwari, Momi, and Walen in West New Guinea. This time the fireflies were different and larger than those seen in Rabaul. They were 7 to 8 mm in length with black wings and they differed in the following respects from the fireflies in Rabaul: (1) The color of the light is bluish in both male and female. (2) The fireflies have a migratory tendency, assembling one by one in a large group, then moving to another tree. Their whereabouts in the daytime is unknown; at any rate they were not on trees.

I also saw synchronous flashing in Singapore on the Bukit timah Road and in the mangrove zone of Johore Baharu, as well as in Java, in certain rice fields in the Village of Provoringo, but these phenomena were on a much smaller scale.

It is said that on the East coast of the Malay Peninsula fireflies that swarm in the mangrove zone used to be protected, since their light could be used for navigation (Watson). Their scientific name is not yet known, but Dr. R. Takahashi believes it to be the *Vesta meneziesi* Motsch.

Synchronous flashing is seldom seen in the Japanese firefly *Luciola cruciata*. At Nagamori Village near Gifu City, Gifu Pref., which is noted as a gathering place, the fireflies swarm on the trees on the river banks and flicker synchronously. However, as the light of *Luciola cruciata* does not extinguish entirely, the regular wave effect, as seen in the tropical species, is not produced. Instead, the flashing is such that the tree on which they are appears to be "breathing" tranquilly.

### Starworm

A luminous insect called *Urat intan* or *Urat bintang* by Indonesians occurs over all tropical Asia. *Urat intan* means the diamond worm and *Urat bintang* the starworm. This animal has a pair of luminous dots on the second and twelfth segments, three luminous dots on each segment from the third to eleventh, and one dot on the last segment. The insect is very beautiful, emitting a bluish green light from each dot. The body is larval in form and wingless, similar to *Phengodes* or the railroad worm of South America (see Harvey, 1952). It is identical with the insect in Sumatra called *Api-Api*, or "fire." I collected it in North Borneo in March, 1938, and afterwards



many of them in Singapore and the Malay Peninsula. The maximum length is 15 mm, and the minimum 3 mm.

On the night of December 15, 1945, at Jurong Village, Singapore, I was observing this starworm in a glass dish on a table under a rubber tree. Suddenly an insect resembling a firefly came flying down. It was about one-third the size of the starworm and had black wings, feather antennae, and no luminous organ. To my great surprise, it copulated with the starworm, and I found that the insect was the male of this species and the full-grown starworm the female. The size of the starworm varies. Some are extremely tiny. Therefore the larva, pupa, and adult female of the starworm sometimes cannot be distinguished. It is not yet known whether the larva of the male or its pupa emits light.

Through the good offices of Dr. Harvey, a specimen was sent to the late Dr. Barber of the National Museum, Washington, who identified it as *Diplocladon Hasseltii*. Dr. R. Takahashi observed that starworms ate millipedes instead of snails.

#### Luminous Fungus Gnat

A most interesting luminous dipteran, belonging to the family Platyruridae, the luminous fungus gnat *Ceroplatus* occurs in Japan.

On September 25, 1948, Mr. T. Shimizu observed the luminous larvae of a fungus gnat living in a web on the fungus *Poria vaporaria*, at Mt. Ryogami in Saitama Pref. The specimens were sent to Dr. T. Esaki (1949) for identification. Larvae hatched from eggs on the way. They were identified as *Ceroplatus nipponicus* Okada (1938). In September, 1950, Dr. Kato and Mr. Shimizu collected some specimens of these insects at the same place. There were two species, one *Ceroplatus nipponicus* and the other *Ceroplatus testaceus* Dalman. According to Dr. Kato (1952), these diptera have two kinds of fat tissue, one consisting of pure fat cells and the other luminous fat cells. The luminous fat tissue is found around the digestive organs. He suggested that the occurrence of luminous fat cells in the fungus gnat indicates a close relation between the luminous substance and the fat metabolism.

One night in June, 1951, on Hachijo Island Mr. Okuyama and I collected luminous larvae of this insect living on a web on the under



surface of the fungus *Ganoderma applanatus*, which grew in a hollow of a big root of the pasania tree (*Shiia Sieboldii*) at Nakanogo Village. The whole body is luminous when it is in the larval and pupal stage, but not in the adult. The larva is 16 mm long and 2 mm wide, with pale brown stripes on a translucent body. The anterior and posterior parts are transparent. The pupa is enclosed in a pure white cocoon knitted by fine threads, cylindrical in form, 15 mm in length and 5 mm in diameter. The transformation from larva to pupa takes one day, from pupa to adult a week. Adults are nonluminous, but the larva and pupa emit a continuous weak, bluish white light. Stimulation does not increase the light intensity. The light of the pupa can be seen through the white cocoon, but the ovary or eggs are not luminous.

I endeavored to culture luminous bacteria from the body and obtained negative results. The luminous larvae were placed in a desiccator containing  $\text{CaCl}_2$  and were dried. This dried material became luminous when moistened with water in the dark, and its light intensity was stronger than that of the living larva or pupa. I tested for the luciferin-luciferase reaction as well as the ATP reaction and obtained negative results.

### Miscellaneous Small Groups

#### Luminous Nemertean

In the summer of 1936 the late Dr. Kanda and I stayed at the Asamushi Marine Biological Station on Aomori Bay. One night, in the aquarium of the station, I recognized luminescence on the surface of a common ascidian, *Chelyosoma siboja*, when irritated. At the same time I collected a luminous nemertean and three species of luminous Polychaeta. These luminous animals were reported on by Kanda (1937) in Japanese.

Among them the most remarkable, the luminous nemertean, was given the new species name of *Emplectonema kandai* by Kato (1939), and was also studied by Kanda in 1939. This animal is from 50 to 120 cm long and only 0.5 to 0.9 mm in diameter. Its body is unsegmented and usually very thin and threadlike, sometimes extending to enormous lengths.

According to my observation the living animal is pale blue in color and glows only when stimulated, emitting a whitish blue light. The light may appear on all parts of the body except the head. No luminous secretion is discharged.

#### Luminous Enteropneusta

Two species of *Balanoglossus* belonging to the family Ptychoderidae occur in Japan. However, the luminosity of these animals has not been observed. They are wormlike in form and about 50 to 60 cm long, live in the sand, and have a disagreeable odor. Recently I observed the luminosity of *Balanoglossus carnosus* (Willey) on the sandy beach in front of the Usa Marine Biological Station of Kochi University, Kochi City. It is very difficult to collect perfect specimens because the body is very soft, especially the posterior part, which can be easily torn off by pulling. I was able to recognize the luminosity of the body and posterior part, even with the head buried in the sand and unexposed. The pale bluish light is emitted from every portion of the body, but only upon stimulation, and luminous slime comes off on the fingers. Another species, *Balanoglossus misakiensis* Kuwano, was found in Misaki, Kanagawa Pref., but I have not yet verified their luminosity. I tested for the luciferin-luciferase reaction as well as the ATP reaction and obtained negative results.

#### Luminous Snake Star

In 1938 the late Dr. Kanda found a luminous snake star in the dredge net at the Mitsui Institute of Marine Biology near Shimoda, Izu Peninsula. This snake star was given the new species name of *Amphiura kandai* by Dr. S. Murakami (1942). The disk of this specimen is 2.5 mm in diameter, and the arms, six in number, are 11 mm in length. According to Murakami, when a few drops of hydrogen peroxide were poured into a dish containing some specimens, the animals emitted light very faintly in the dark. Kato (1947a) also studied the luminous cells of this animal and surmized that the light is intracellular.

#### Bryozoa

Kato (1950) reported on the luminous organ of *Acanthodesia serrata* (Syn. *Membranipora membranacea*), a common Japanese marine

bryozoan. He noted that the bryozoan colony emitted a bluish light for a few seconds when stimulated. A pair of light organs are situated on each anterolateral part of the ventral membranous area in each zoecium.

### Pisces

Luminous fishes, depending on their method of light production and the type of luminous organs, are divided into three groups: (1) fish associated with luminous bacteria, (2) fish with well-developed luminous organs or simple luminous skin organs, (3) fish with indirect emission luminous organs, that is, the luminous gland lies inside the fish body and the light is reflected so as to pass through a translucent area of muscles.

#### Fish Associated with Luminous Bacteria

In Japan the first group of luminous fishes have been studied in rather great numbers. In 1916 Dr. Harvey, during his stay in Japan, saw the light organs of the knightfish *Monocentris japonicus*, and he predicted that luminous bacteria would be found in the luminous organs. Later Yo. K. Okada (1926) studied the morphology and histology of the organs, and Yasaki (1928) cultivated luminous bacteria from the organs and confirmed Dr. Harvey's prediction. This fish is commonly found in shallow water along the coast of Japan. The fish is a beautiful golden yellow in color. Its luminous organs consist of two oval protuberances lying side by side at the tip of the lower jaw. In a fish 12 cm long the light organ is 4 mm long and 3 mm wide.

The fishes of the families Gadidae and Macrouridae possess luminous glands on their ventral surfaces. The Gadidae live in comparatively shallow water and may be caught close to the coast of Japan. The Macrouridae are deep water fish and are always caught by trawlers as they inhabit a region over 100 fathoms deep. They are taken most abundantly during winter season along the Pacific coast of Japanese waters.

Kishitani (1930) examined the luminous duct of *Physiculus japonicus* of the Gadidae and discovered it was an open type of luminous gland containing symbiotic luminous bacteria, *Micrococcus physiculus* Kishitani.

Dr. Yasaki and I (1936) had reported that there were 10 species of the Macrouridae, which are closely related to the Gadidae and have a luminous organ of the same type. I (1938-1951) have added one species of the Gadidae and 4 species of the Macrouridae and I was able to obtain several strains of luminous bacteria from each species of the Gadidae and Macrouridae. These strains of bacteria were obtained from various species of fish caught at different times and in different localities. All the bacteria had the same general biological characteristics but they varied in their optimum temperature, being higher in some cases but never varying greatly. I think all these luminous bacteria are of the same group.

#### Fish with Indirect Emission Luminous Organ

The fishes of the families Acropomatidae and Leiognathidae possess luminous glands containing symbiotic luminous bacteria. These fishes can also be classified under the third group since the luminous gland lies inside the body and the light therefore passes through a translucent area of muscles.

*Acropoma* is a genus of fish of the family Acropomatidae found in the southern Sea of Japan. *Acropoma japonicum* Gunther is known as *Hotaru-jako* in Japanese, meaning firefly small fish, and is considered there to be a single species. However, I have observed that the luminous organ varies in shape and position in two types of fish. For this reason they may represent different species.

Recently Matsubara (1953) reported another new species named *Acropoma hanedai* Matsubara, which I (1950) had mentioned as the second type of *Acropoma*. During the winter season they occur in southern Japan as a mid-water dweller, in depths ranging from about 80 to 200 fathoms. They are a beautiful pink in color and attain a length of 200 mm. There is no difficulty in obtaining specimens in the Mimase fish market near the city of Kochi, Shikoku, Japan.

These fish differ from other luminous fish in possessing an unusually large luminous area. In fact, the lower part of the muscles of their entire body surface is utilized for this purpose.

The diffused light comes from a luminous U-shaped filiform body in the muscle tissue not visible from the outside. It consists of the luminous gland, white reflector, lens, and an opening near the anus

with a long duct which connects with the luminous gland. The luminous gland of *Acropoma hanedai* is very long compared with that of *Acropoma japonicum*, and the muscles which form the lenses are comparatively poor. I obtained pure cultures of luminous bacteria from both species of *Acropoma* and found their general biological characteristics quite different.

Other luminous fishes of the same general type as *Acropoma* belong to the Leiognathidae. These are true shallow-water forms and are abundant in southern Japanese waters and in tropical seas. In 1937 at Palao I observed that *Gazza minuta* and several other species of this family are luminescent when alive. Externally this fish does not present any unusual features. It was only by careful observation of the living fish by night that the luminosity of the lower half of the body was revealed.

The source of the luminosity is a swollen ring of gland which encircles the esophagus. The body cavity and the thoracic and ventral muscles are so modified as to increase the efficiency of the light-producing mechanism. The light is visible externally as a diffused greenish blue light, sometimes intermittent. The light control mechanism is due to chromatophores which are scattered over the transparent membrane that covers the luminous gland. These fishes have a far more complex luminous organ than *Acropoma*. In Japan there are three species, *L. argentum*, *L. rivulatum*, and *L. elongatum*, which are usually dried for sale for food. In more tropical countries there are many species of Leiognathidae. I obtained and observed eleven species of Leiognathidae, distributed in three genera (*Leiognathus*, *Secutor*, and *Gazza*) in the southwestern Pacific.

*Gazza* occurs in clear water in Palao at depths of about 30 meters, while *Leiognathus* lives in turbid water in the mangrove zone at depths of 1 to 2 meters. In Palao, *L. equulus* ranged in length up to 70 mm, while in Sandakan Bay, North Borneo, it was 200 mm long. In Sandakan, Chinese fishermen catch these fish in nets and bring them for sale to the Sandakan fish market.

Around Singapore and the Malay Peninsula these fish are easily obtained in all seasons from fishing traps known as "*Kelong*." In 1938 Fowler reported twelve species of this family in Malaya. In Java I collected many species of this family in the fish markets of Jakarta.



An interesting feature of the Japanese species *L. rivulatum* is that the luminous gland of the male is either very large or very small. I am of the opinion, though by no means certain, that this difference in size is due to age. The luminous gland of the female is smaller than that of the male and therefore gives off a weaker light.

I was able to obtain pure cultures of luminous bacteria from various species of the fish caught at different times in Japanese and tropical waters. They had the same general biological characteristics,

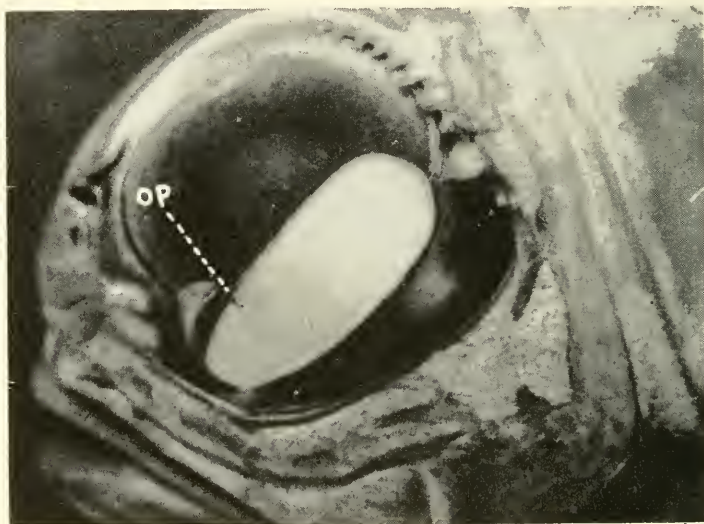


FIG. 9. The eye of the luminous fish *Anomalops*, showing the elliptical white luminous organ with its opening (O P).

with only slight variations, probably due to the effects of temperature. All these luminous bacteria appear to be of the same group.

The famous luminous fish *Anomalops* is found not only in the East Indies but also in the South Pacific Islands, the New Hebrides, Fiji, and Paumotu. This fish has a large half-moon shaped luminous organ below the eye, as shown in Fig. 9. The back of the organ is covered with a layer of black pigment. *Anomalops* is able to shut off its display of luminescence at will, by rotating the organ, so that the luminous part is turned away from sight and the black nonluminous back takes its place.



In June, 1942, I saw schools of *Anomalops* at the surface of the water in the harbor of Manukuwari on the northwest coast of New Guinea. At that time I collected three small specimens and was able to observe their luminescence. When this fish is swimming under natural conditions, the luminous surface appears and disappears intermittently. If the fish is caught and put into a glass jar, its luminous display becomes irregular, if the water is in any way unsuitable. A fish in a dark place which is suddenly illuminated by switching on

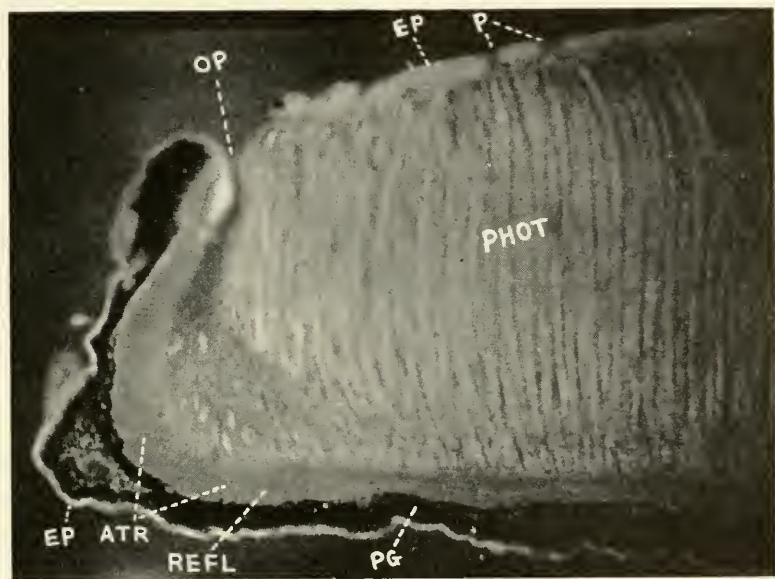


FIG. 10. A longitudinal section of the luminous organ of *Anomalops*. OP, opening; EP, outer epithelium; P, pores; PHOT, luminous ducts; REFL, reflector; PG, pigment; ATR, artery.

an electric light will cease to display luminosity in one or both organs. In daylight the fish will not display its luminosity, but if the place in which it is kept is suddenly darkened, its luminosity is immediately displayed and appears as a bluish green light.

It was very remarkable that 4 specimens of this fish were obtained near Japanese waters. The first specimen was caught in the sea near Kominato, Chiba Pref., Japan, and was recorded by Abe (1942). The second specimen was caught with hook and line near Kanminato Bay, Hachijo Island, and was also reported by Abe (1951). The third and

the fourth specimens I found among some preserved fish materials at the Hachijo Branch of Tokyo-to Fisheries Experimental Station, but the date of collection is unknown. The total length of the third specimen was 190 mm; the diameter of the eye was 20 mm, but the luminous organs had putrified. The total length of the fourth specimen was 145 mm; the luminous organ was 14 mm long, 5.5 mm wide, and 1 mm thick. In a parallel section of the organ (Fig. 10) a large number of glandular tubes can be seen parallel to each other and extending completely across the organ from the back pigmented surface to the front transparent surface, with blood vessels running between them. A cross section of the organ is shown in Fig. 11.

Harvey (1925) suggested that the luminescence is due to symbiotic luminous bacteria. He cultured bacteria from these luminous organs, but these cultured bacteria were not luminous.

In a former report (1942) I noted that my culture test from the organs agreed with the result of Harvey's test, although I was doubtful of his bacterial theory. I had observed that the section of the organ was quite different from those in other symbiotic luminous fishes, *Monocentris*, *Physiculus*, *Malacocephalus*, *Acropoma*, and *Leiognathus*, and that the organ was closed, that is, it had no duct to the exterior. However, upon investigation of the large specimens of Hachijo Island, I was able to find the opening from the duct in the luminous organ, which I had overlooked in my former report. The opening to the exterior is considered to be the small depression in the dorso-anterior part of the luminous organ. There are also many pores scattered over the surface of the organ. Therefore the organ is of the open type and supports the bacterial theory of light emission, especially since the luminescence is continuous.

#### Self-Luminous Fish

The second group of luminous fish, namely fish with a well-developed luminous organ or simple skin photophores, were reported by such ichthyologists as Tanaka (1908, 1912), Ishikawa (1915), Nakasawa (1932), Kamohara (1936, 1940, 1952), Matsubara (1936, 1938, 1950), Abe (1942, 1951), Imai (1942), and Kuroda (1950). However, most of these are taxonomical reports.

Recently Abe and Nakamura (1954) reported an adult female, with

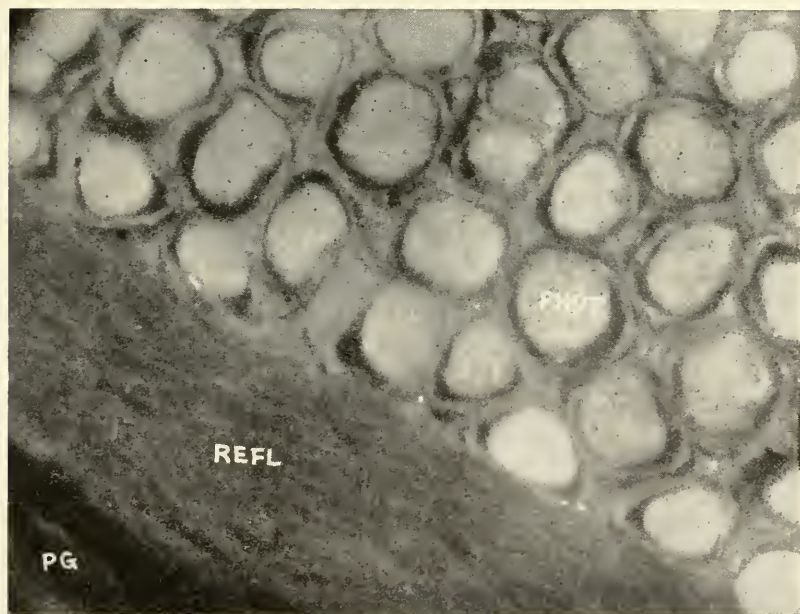
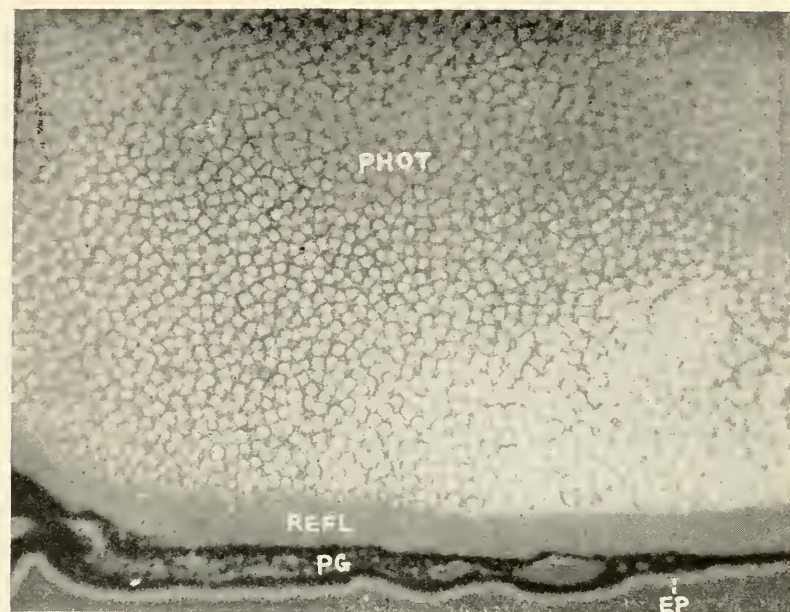


FIG. 11. Transverse section of the luminous organ of *Anomalops*. Lettering same as in Fig. 10.

a supposedly parasitic male, of the deep sea angler fish *Cryptosparus couesi*, from the Pacific coast of northern Japan which is supposed to be luminous.

The structure of the light organs and the observation of luminous phenomena of self-luminous fish have been investigated by Ohshima (1911). He studied two species of deep sea luminous shark and some specimens of the Myctophidae and Sternoptychidae. In my observation (1950) on living material of *Yarrella* and *Polyipnus* from Suruga Bay, I recognized a peculiar color filter, hitherto considered a lens, situated between the luminous tissue and the lens. Recently I observed luminescence of the cheek organ of a deep sea luminous fish, *Astronesthes iijimai* Tanaka (1908), which was collected in a shrimp trawl net in Suruga Bay. This fish has a cheek organ and two rows of minute photophores along the ventral and lateral walls. The structure of the cheek organ is very similar to that of *Anomalops*. Although luminescence is continuous, the luminous surface appears and disappears at will by rotating the cheek organ as in the case of *Anomalops*. Comparative studies of the structure and substance of both luminous organs should prove interesting. We may find luminous bacteria and a close relationship between the two organs. As deep sea luminous fish are caught with comparative ease by trawlers in many localities in Japan, it is to be expected that many unrecorded luminous species will be found in the future.

Dr. A. Terao and others (1950) reported a luminous flying fish which was collected from Sagami Bay, Japan. They described the luminous organs as minute, weak, luminescent points scattered on the dorsal surface of the fish and rather strongly luminescent points on the head. The former resemble the skin organ of a luminous shark, and the latter are like the organs of *Monocentris*. However, I observed several living species of flying fish in the dark at Hachijo Island and Suruga Bay, but could not recognize any luminous points on the surface.

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## Ecology of Autotrophic Marine Dinoflagellates with Reference to Red Water Conditions\*

JOHN H. RYTHER

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

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Although dinoflagellate luminescence was at one time believed to be restricted to a few highly specialized forms (i.e., *Noctiluca*, *Pyrocystis*), as early as 1830 Michaelis described 9 luminous species of the common genera *Ceratium*, *Peridinium*, and *Prorocentrum*. Kofoed and Swezy (1921) attribute this property to "many if not most of the Peridiniales and Gymnodiniales." The extent to which bioluminescence occurs in the group as a whole has not been systematically investigated, but the opinion now appears to be widespread among workers in the field that most dinoflagellates, at least under certain conditions, exhibit luminescence. Kofoed, in a personal communication to Harvey (1952), went so far as to suggest that perhaps all dinoflagellates have this property.

The dinoflagellates are found in all the oceans of the world and at least a few are usually present at every time of the year. Among all forms of marine life, they are probably second in abundance only to the diatoms and, in many cases, may greatly outnumber them. Together with the diatoms they constitute the bulk of the so-called phytoplankton, the community of unicellular, autotrophic organisms that are the basis for all life in the sea.

In view of the widespread occurrence of dinoflagellates in the ocean and the apparent preponderance of luminous forms, this group must be considered as one of the principal producers of biolumines-

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cence in the sea. Allen (1939) stated categorically that they are the most common cause of this phenomenon. It follows that any consideration of the factors contributing to the occurrence of marine bioluminescence must include a consideration of the ecological factors that influence and control the growth and distribution of its principal causative agents, the dinoflagellates. The following discussion will be concerned with the general ecology of these organisms. In addition, special attention will be devoted to a possible explanation for the frequently reported occurrences of dense "blooms" or "swarms" of dinoflagellates in many parts of the world.

No attempt will be made in the following discussion to differentiate between luminous and nonluminous dinoflagellates, but it will be assumed that the presence or absence of this property will not appreciably affect the interrelationships between the organisms and their environment. A consideration of their ecology as a group is complicated, however, by the fact that individual species may employ almost any type of nutritional habit, including autotrophic, holozoic, saprophytic, and parasitic. Although the number of species which employ the second mode of nutrition, either obligatorily or facultatively, is apparently quite large (Kofoid and Swezy, 1921), in total abundance they are probably insignificant in comparison with the photosynthetic forms. For this reason we will consider here primarily the autotrophic dinoflagellates.

### General Ecology of Dinoflagellates

#### Temperature

Dinoflagellates are found in all parts of the ocean and are usually present in some quantity at all times of the year. It is therefore impossible to make broad generalizations concerning the temperature relationships of so large and widespread a group. Some species are obviously arctic forms and may be observed living in subzero temperatures associated with the polar seas (Gran, 1924; Braarud, 1935). However, there does appear to be some justification for classifying the great majority of the dinoflagellates as warm-water organisms. This is particularly true if they are compared as a group with the diatoms, which, in contrast, are often considered cold-water forms.



In the temperate regions of the ocean, the relatively unproductive winter season is normally followed by a spring "flowering" or "bloom" of phytoplankton, which consists predominantly of diatoms. This spring maximum, which usually develops into the largest population of the year, becomes limited by the supply of available nutrients and may pass through its entire cycle of growth and decline in a few weeks. As the nutrient level falls and the temperature rises, the numbers of diatoms decrease and many species may disappear entirely from the plankton.

By late spring, the dinoflagellates appear in significant numbers, and while they seldom attain the abundance of the spring diatom bloom, they often persist as the dominant member of the plankton community throughout the summer months.

The sequence of events described above occurs in temperate regions in the open ocean (Herdman, 1922), in coastal and slope waters (Gran and Braarud, 1935), and in estuarine situations (Gaarder and Gran, 1927; Marshall, 1947; Braarud, 1945).

While northern waters support larger populations of dinoflagellates, the number of temperate species is extremely small compared with the number of tropical forms (Sverdrup, Johnson, and Fleming, 1942). This is particularly true of the highly specialized Dinophysiales, which are characteristic of tropical seas (Fritsch, 1935) but also applies to the ubiquitous genus *Ceratium* of which Peters (1932) found 33 of 55 South Atlantic species confined to warm water.

The dense populations of dinoflagellates which create "red water" conditions are known only in the tropics or in temperate water during the warmer (and usually the warmest) time of the year. This subject will be discussed in more detail in another section. Dinoflagellate luminescence shows a similar seasonal periodicity in the temperate parts of the ocean. Allen (1939) reported that luminous displays by these organisms are common in the La Jolla region during the summer months, but never occur between October and May.

Such fragmentary physiological evidence as is available concerning the temperature relations of dinoflagellates appears to support the view that they are predominantly a warm-water group. Barker (1935), who is one of the pioneers in developing successful culture methods for dinoflagellates, observed optimal temperatures for the growth of

some 14 species between 18° and 25° C. Braarud and Pappas (1951) noted a temperature optimum for *Peridinium triquetrum* at 18° C, while Nordli (1953) found that *Ceratium fusus* and *C. furca* grew most rapidly at temperatures of 15° and 20° C respectively. Provasoli (personal communication) finds temperatures of 20–25° C most suitable for growing *Gyrodinium californicum*. Although the preceding experimental evidence is obviously insufficient to form a basis for generalization, it is perhaps significant that all the above species, despite their relatively high temperature optima for growth, were isolated from temperate regions of the ocean.

### Salinity

Dinoflagellates occur in fresh, brackish, and full sea water, and it is again impossible to define optimal or limiting salinity conditions for the group as a whole. The unarmored dinoflagellates appear to be most abundant in the open ocean plankton, while the armored forms are more typical of coastal and estuarine regions (Fritsch, 1935). This suggests that the optimal growth conditions for the latter may occur at somewhat lower salinities than are observed in the open ocean.

Several plankton investigations have revealed that dinoflagellate maxima may often be correlated with the seasonal or geographical occurrence of relatively low salinity water (Gran, 1924; Marshall, 1947; Gaarder and Gran, 1927; Marshall and Orr, 1927; Gran and Braarud, 1935). It is noteworthy that the majority of such studies have been concerned principally with the armored species and have largely neglected the smaller, naked dinoflagellates owing to the difficulties involved in collecting and preserving them.

Nordli (1953) found optimum salinities for the growth of three species of *Ceratium* (*C. furca*, *C. fusus*, and *C. tripos*) at 20–25‰. He was able to correlate the relatively low salinity and high-temperature optima of these forms with similar conditions in the regions where they occur along the Norwegian coast. Nordli suggests that the so-called Tripos-plankton region of Gran (1902) off the northern coast of Norway may be a biogeographical area limited by high-temperature and low-salinity borders.

Table I gives a summary of the optimum and maximum range of

TABLE I  
Optimum and Maximum Range of Salinity for Growth of Some  
Neritic Dinoflagellates

Reference	Species	Optimum, ‰	Range, ‰
Nordli (1953)	<i>Ceratium furca</i>	25	10-40
	<i>Ceratium tripos</i>	20	10-35
	<i>Ceratium fusus</i>	20	10-40
Braarud (1951)	<i>Amphidinium</i> sp.	15	5-45
	<i>Exuviella baltea</i>	18	5-35
	<i>Peridinium trochoideum</i>	20	5-60
Braarud and Rossavik (1951)	<i>Prorocentrum micans</i>	15-20	10-45
Braarud and Pappas (1951)	<i>Peridinium triquetrum</i>	15-20	10-40

salinity for the growth of several species of common neritic dinoflagellates as determined by Braarud and his co-workers.

While all the species have salinity optima well below that of full sea water, it is perhaps of even greater significance that they are also able to grow within an extremely wide range of salinities. This high degree of adaptability is a definite advantage to life in the variable environment of coastal and estuarine waters, and it is perhaps one of the means by which the neritic dinoflagellates are able to compete successfully with other organisms, such as diatoms, which in general have a more narrow range of salinity tolerance.

#### Nutrient Requirements

The autotrophic dinoflagellates, as other members of the phytoplankton, are dependent upon dissolved mineral salts for their nutrition. According to Vinogradov (1935) the peridiniins contain approximately the same relative concentrations of nitrogen, phosphorus, calcium, and iron as does sea water.

The dinoflagellates have often been credited with the ability to utilize and flourish in extremely low concentrations of nitrogen and phosphorus (Gran, 1926-27; Gilson, 1937). This concept has stemmed largely from observations that dinoflagellate maxima, in temperate

waters, follow the decline of the spring diatom flowerings and relatively large populations often persist throughout the summer months when the supplies of these nutrients are almost undetectable. Gran (1926-27) has proposed that the dinoflagellates require less nutrition for growth than the diatoms on account of their relatively low rate of metabolism.

There is some experimental evidence that dinoflagellates can utilize nitrogen and phosphorus at rather low concentrations. Thus Barker (1935) observed that increasing the nitrogen content of aged sea water by 1000 to 10,000 times did not increase the growth rates of *Prorocentrum micans*, *P. gracile*, or *Peridinium* sp. Similarly *P. micans* grew equally well in  $K_2HPO_4$  concentrations ranging from  $5 \times 10^{-6}$  to  $5 \times 10^{-3}\%$ . King (1950) found that increasing nitrogen and phosphorus 1 to 200 times their concentrations in aged sea water did not increase the growth rate of *Gymnodinium simplex*.

There is no indication, however, that such low nutrient concentrations are necessary for the optimum growth of these organisms. Braarud (1945) found that maximum populations of several dinoflagellates occurred in the regions of heaviest pollution in the Oslofjord. Marshall (1947) observed a dense growth of *P. triquetrum* in Loch Craiglin immediately following fertilization of the loch. In the following section, a factor other than the nutritional physiology of the dinoflagellates will be discussed as a possible explanation for their occurrence and growth in nutrient-poor waters.

As mentioned earlier, dinoflagellates as a group show a continuous variation in their modes of nutrition from autotrophic to holozoic, while many species are facultative, obtaining their food by either or both methods (Kofoed and Swezy, 1921). We are concerned here principally with the former type of nutrition. However, it is perhaps questionable whether any of the dinoflagellates are completely autotrophic, in the literal sense of the term.

For many years they have remained among the most difficult of marine organisms to grow and maintain in culture. The author knows of no case in which dinoflagellates have been grown in a completely inorganic medium, either of the completely artificial or the enriched sea water type. The few media which have been developed and used successfully for growing these organisms include, almost without

exception, soil extract as their common ingredient (Barker, 1935; Gross, 1937; Braarud, 1951; Sweeney, 1951).

Sweeney found that this substance best supported the growth of *Gymnodinium splendens* when aged for 4–6 weeks, and it was relatively ineffective when freshly prepared or aged for more than two months. Neither Barker nor Sweeney was able to replace soil extract with trace element mixtures. The latter author concluded that the active ingredient is probably organic in nature, and later (Sweeney, in press) succeeded in replacing soil extract with vitamin B<sub>12</sub>.

King (1950) cultured *Gymnodinium simplex* in a medium consisting of McClendon's artificial sea water, Hoagland's trace element mixture, and yeast extract. Again, she was unable to obtain growth with the inorganic constituents alone, but was able to replace yeast extract with a mixture of amino acids added as pure chemicals in the same ratio as they occur in the yeast extract.

Provasoli and Pintner (1953) synthesized an artificial medium for the growth of *Gyrodinium californicum* which contained, in addition to the common inorganic nutrients and trace metals, a chelating agent (EDTA), NaH glutamate, *dl*-lysine, *dl*-leucine, and vitamin B<sub>12</sub>.

Although the growth-promoting ingredients of soil extract have not been identified, it would appear likely, in view of the preceding evidence, that they include growth factors such as vitamin B<sub>12</sub>. The organisms may also derive some benefit from humic acid and other ingredients which may act as chelating agents, reducing the concentrations of some one or more of the trace metals to a nontoxic or noninhibitory concentration.

In inshore waters dinoflagellates are often abundant, and in many parts of the world their populations may develop bloom proportions. Here the close association of the plankton with land masses and the contribution of runoff water to their environment provide what may be considered as a natural "soil extract," and, in the vicinity of heavily populated areas, frequently a source of organic pollutants. As mentioned earlier, Braarud (1945) observed a heavy growth of dinoflagellates in the highly polluted regions of the Oslofjord. Braarud and Pappas (1951) later found that the addition of small amounts of raw sewage to the medium stimulated the growth of *Peridinium triquetrum*.



In the open ocean, the presence of free organic compounds is more difficult to account for. In this case it is perhaps the metabolites or decomposition products of other plankton organisms which create the necessary growth conditions for the dinoflagellates.

It has been pointed out that many of the dinoflagellates found in temperate waters have relatively high-temperature optima, a fact which may explain their paucity in the winter plankton community and the spring blooms. However, there are certainly many species which are able to show some growth in temperatures of 0° C and below (Braarud, 1935). Yet even these are conspicuously absent from the usual spring flowering of diatoms. Thus temperature alone may not be sufficient to account for the seasonal periodicity of the dinoflagellates. It would appear that, in many cases, the growth of these organisms may be also dependent upon, or at least benefited by, the previous existence of a flowering of diatoms.

The underlying cause for this type of succession is probably nutritional, but its exact mechanism is obscure. The diatoms may reduce the concentrations of one or more of the nutrients or trace metals to a level favorable for the growth of the dinoflagellates. This type of relationship was proposed as an explanation of the succession of plankton elements in freshwater by Pearsall (1932) and Hutchinson (1944). A somewhat different type of relationship has been suggested by Lucas (1947, 1949), who has proposed the production of external metabolites or "ectocrines" by one group of plankton organisms which may have a beneficial effect upon the succeeding population and an inhibitory effect upon other competing organisms. This has been demonstrated in fresh water by Rice (1954).

### Ecological Significance of Motility in Dinoflagellates

The presence of motility in the dinoflagellates, and its absence in the diatoms, may have an important bearing in the relationships of these organisms with such environmental conditions as temperature, salinity, and the nutrient concentration of the water. This is the hypothesis of Gran (1926-27) and is supported by Braarud (1935).

The diatoms are dependent upon vertical mixing and their natural buoyancy for remaining in the upper, photosynthetic zone of the ocean. They may derive some benefit from the synthesis of fats and



its inclusion as oil droplets in their protoplasm and may possess structural adaptations of various types which increase their surface area and hence retard sinking (Gran, 1912; Russell, 1927; Sverdrup, Johnson, and Fleming, 1942). Gross and Zeuthen (1948), on the other hand, attribute the buoyancy of diatoms to their ability to maintain extremely low concentrations of divalent ions in their cell sap. According to these authors, under suitable physical conditions plankton diatoms have a specific gravity equal to that of sea water and do not sink, but at temperatures of 20° C or above, this equilibrium does not exist and the cells settle to the bottom. In general diatoms may be said to thrive in cold, fully saline ocean water at times of the year when there is considerable mixing of the surface water.

In temperate or northern summer conditions, the density of the water decreases as its temperature rises. For example, an increase in temperature from 0–25° C decreases the viscosity of sea water by one-half (Sverdrup, Johnson, and Fleming, 1942). In addition, vertical mixing is usually at a minimum in summer. Under these conditions, the diatoms find it increasingly difficult to remain in the upper water layers, and in Gran's opinion, their populations are unable to maintain themselves in the euphotic zone.

In the tropics, this situation usually prevails throughout the year and provides a definite disadvantage to the diatoms. For the same reason, they may encounter suboptimal conditions in the low-salinity (and hence low-density) waters of many coastal and estuarine situations.

The dinoflagellates, on the other hand, possessing the advantage of motility, are able to maintain themselves in water of low density with comparative ease and are relatively independent of vertical mixing. Furthermore, they, too, are often assisted by morphological adaptations which increase their surface area and thereby retard sinking. In the tropical forms, these structures far surpass similar features of the diatoms. Thus *Ceratium* is reputed to have the ability of prolonging or shedding its horns as it moves into warmer or colder water masses (Fig. 1). Others, like *Ornithocercus*, grow wide, wing-shaped membranes (Fig. 2). Some species, such as *Triposolenia*, have asymetrically arranged appendages so that, as soon as they stop swimming and start to sink, they are quickly oriented horizontally in the water,

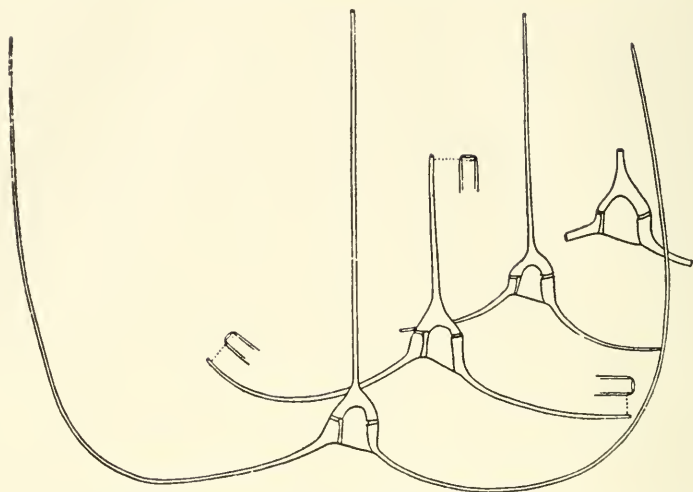


FIG. 1. *Ceratium trichoceros*, showing progressive and proportionate reduction of the horns in autotomy. (After Murray and Hjort.)

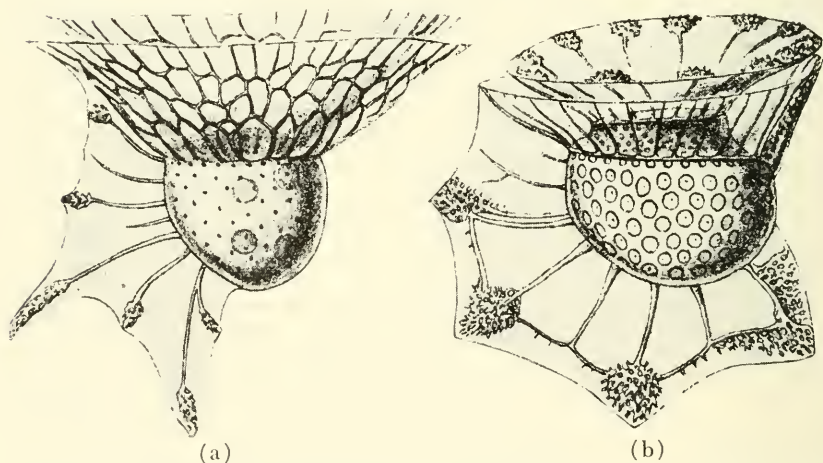


FIG. 2. (a) *Ornithocercus splendidus*; (b) *Ornithocercus steinii*. (After Murray and Hjort.)

providing a maximum surface as resistance to sinking (Kofoid, 1906). Thus the dinoflagellates, in contrast to the diatoms, appear to be particularly well adapted for life in relatively calm, low-density water.

In addition, and of perhaps greater significance, the same factor of

motility provides the dinoflagellates with an advantage in waters of low nutrient content. The nonmotile diatoms are dependent upon the dissolved nutrients contained in the water which immediately surrounds them and through which they sink (see Munk and Riley, 1952). In contrast, the dinoflagellates, though not strong swimmers, can move about for considerable distances and localize in the most advantageous depth for photosynthesis. If the nutrient level of the water is low, they may, by their vertical migrations, utilize all the nutrients available within the entire photic zone. According to Peters (1929) *Ceratium* can move through 5 to 10 meters in 12 hours or less.

Thus motility may be equally as important as the basic physiological characteristics of dinoflagellates in providing the means for their existence in waters of high temperature, low salinity, and reduced nutrient concentration.

### **“Red Water” Phenomena**

#### **Background**

In certain parts of the world, usually in coastal or estuarine regions, dinoflagellates either grow or aggregate to such an extent that they impart a distinct coloration to the water. Such manifestations are known popularly as “red tide” or “red water,” since the predominant color is of a reddish hue, although yellow or brown dinoflagellate blooms are not uncommon.

According to Allen (1946) a cell concentration of one-half to one million organisms per liter may give a chocolate-brown color to the water, while a doubling or trebling of this number is sufficient to produce red water. Concentrations of over 50 million cells per liter are not uncommon in patches of red water (Woodcock, 1948; Davis, 1948).

Frequently these aggregations of dinoflagellates are accompanied by a mass mortality of marine organisms (Whitelegge, 1891; Aiyar, 1936; Gunter *et al.*, 1948; Connell and Cross, 1950). However, many red water reports make no reference to such calamities. In many instances these outbursts are also accompanied by brilliant displays of luminescence.

The reported occurrences of red water are too numerous to review

TABLE II  
Summary of Some Occurrences of "Red Water" Conditions in Various Parts of World  
(M = mass mortality, L = luminescence)

Reference	Location	Time	Organism(s)	Ecological conditions
Whitelegge (1891)	Port Jackson, Australia	March, 1890	<i>Glenodinium rubrum</i>	Hot weather following heavy rainfall. Occurred in streaks or patches. (M)
Nishikawa (1901)	Bay of Aomori, Japan	Sept., 1900	<i>Gonyaulax polygramma</i>	Occurred in patches, concentrated at surface. Floated on surface of container. (L)
Hirasaka (1922)	Gokasho Bay, Japan	Dec.-March, 1921	<i>Gymnodinium sanguineum</i>	Entire bay chocolate or red color. Restricted to layer approx. 1 meter thick. Diurnal vertical migrations. (L)
Ritchie (1952)	South coast of Honshu	May, 1950	<i>Noctiluca</i> sp.	Occurred in streaks offshore. Driven onshore by southerly winds. (L)
Hornell and Nayudu (1923)	Malabar coast, India	Annually, Aug.-Sept.	peridinians	Follow southwest monsoons, diatom blooms, and heavy rainfall. Occur during calm, hot weather. In bands along shore or patches which moved with tide.
Aiyar (1936)	Madras coast, India	June, 1935, and other years	<i>Noctiluca miliaris</i>	Follow diatom blooms in hottest time of the year. Salinities 34-35‰ (M)
Menon (1945)	Trivandrum coast, India	Annually, June-Sept.	<i>Gymnodinium</i> sp.	Follow rains and diatom maxima.
Bhimacher and George (1950)	Malabar coast, India	Oct., 1948	<i>Noctiluca miliaris</i>	After southwest, before northeast monsoon; calm, hot weather; salinity ca. 35‰/‰.

Gilchrist (1914)	Walvis Bay, So. Africa	Annually in December	<i>Noctiluca</i> sp.	Follow periods of offshore wind. (M, L)
Brongersma-Sanders (1948)	Walvis Bay, So. Africa	Annually in Dec.	<i>Noctiluca</i> sp.	During periods of minimal upwelling. Water temperatures highest of year. (M, L)
Allen (1946 and ref.)	California coast	Frequently in midsummer	<i>Gonyaulax polyedra</i> <i>Prorocentrum micans</i>	Usually during periods of calm, hot weather, smooth seas. (L)
Bonnot and Phillips (1938)	Monterey Bay, Calif.	Aug.-Sept., 1937	<i>Gonyaulax catenella</i>	Developed inshore, spread seaward in bands. (L)
Torrey (1902)	San Pedro, Calif.	July, 1902	<i>Gonyaulax</i> sp. <i>Noctiluca</i> sp.	Occurred in patches of same temp. and salinity as surrounding seawater. (M, L)
Gunter <i>et al.</i> (1948)	West coast of Florida	Nov., 1946-Aug., 1947	<i>Gymnodinium brevis</i>	Calm, hot weather, preceded by hurricane and heavy rains. Temperature 24-27° C
Chew (1953)	West coast of Florida	Nov., 1952	<i>Gymnodinium brevis</i>	salinities 36-37‰ (M) Occurred in patches. Temperature 23-25° C, salinity 33-34‰
Connell and Cross (1950)	Offats Bayou, Texas	Midsummer, 1949	<i>Gonyaulax</i> sp.	Calm, hot weather following heavy rainfall. (M, L)
Lund (1936)	Texas coast	June-Aug., 1935	?	Calm, hot weather following heavy rainfall
Martin and Nelson (1929)	Delaware Bay, N.J.	July-Aug., 1928	<i>Amphidinium fusiforme</i>	Clear, fine weather. Occurred in bands. Temp. 25-28° C.
Mead (1898)	Narragansett Bay, R. I.	Aug.-Oct., 1898	<i>Gymnodinium</i> sp. <i>Peridinium</i> sp.	Calm, hot weather. (M)
Marshall and Orr (1927)	Loch Striven, Scotland	June, 1925	<i>Peridinium triquetrum</i>	Period of high temperature, and low salinity.
Braarud (1945)	Oslofjord, Norway	Aug., 1938	<i>Peridinium triquetrum</i>	In polluted part of fjord, concentrated at surface.

here completely. Table II gives a partial review of the red water literature, including the time and place of the occurrence, the causative organism, and a brief statement of the ecological conditions preceding or accompanying the outbreak, in so far as these have been observed.

### Prerequisites for Red Water Conditions

The basic requirements for an outbreak of red water may be summarized under the following three headings: (1) a seed population of dinoflagellates, (2) the existence of favorable conditions for the growth of one or more of the species present, and (3) either the concentration of sufficient nutrients to permit the dense growth of the organisms, or the concentration of the organisms themselves, to the degree to which they are found in "red water."

The presence of a seed population of dinoflagellates is a condition which is probably always met, for there are at least a few of these organisms in almost every part of the ocean at all times. However, this is undoubtedly an important factor, together with the environmental conditions, in determining the particular species of dinoflagellate dominating a red water outbreak. Thus, it is somewhat more difficult to account for the origin of *Gymnodinium brevis*, the causative agent of the Florida "red tide," which appears to be present in the region only during its periods of blooming. Slobodkin (personal communication) is of the opinion that seed populations of this organism are maintained in the brackish to freshwater regions of the Florida Everglades.

In maximum developments of "red water," the optimal growth conditions for the species involved are perhaps met or closely approached. However, such ideal situations are probably less common than the existence of an environment in which the growth of some one species of dinoflagellates is favored over that of all other phytoplankton forms. In most parts of the ocean the diatoms are the principal competitors of the dinoflagellates, though other forms, such as Chrysophceae, Chlorophyceae, and Euglenineae may be important in estuarine conditions.

In the preceding sections of this report we have seen that the dinoflagellates can compete successfully with the diatoms under conditions of high temperature, low salinity, and reduced nutrient



concentrations. They appear to require the presence of some one or more unknown organic substances, and there is some indication that they benefit from the previous existence of a large population of diatoms. It is doubtful if all these conditions are usually satisfied at any one time, and unlikely that they are all necessary for the dominance of dinoflagellates.

An examination of Table II reveals one factor which is almost universal in red water outbreaks, the occurrence of a high water temperature. In temperate or boreal regions of the ocean, red water appears to be restricted to the summer months, and the notation is frequently made that it is preceded by periods of unusually hot, calm weather. Along the Indian coast, red water occurs during the clear, hot periods between the southwest and the northeast monsoons (Hornell and Nayudu, 1923; Menon, 1945; Bhimachar and George, 1950). Off the Peruvian and Southwest African coasts it appears during the southern summer when the upwelling of cold water is at a minimum and water temperatures are the highest of the year (Brongersma-Sanders, 1948). Allen (1946) described a number of occurrences of red or yellow water along the California coast which have always occurred in mid-summer during periods of hot, calm weather and smooth seas.

There is less evidence that low salinity is an important factor in red water outbreaks. Slobodkin (1953) has shown a close correlation of red tides along the west coast of Florida with previous periods of exceptionally heavy rainfall. He has proposed that the organisms develop in small, discrete masses of relatively low-salinity water which result from the increased land drainage during heavy rains and are maintained in the ocean by density gradients. Table II gives several other instances in which red water has been preceded by heavy rainfall (Whitelegge, 1891; Hornell and Nayudu, 1923; Lund, 1936; Menon, 1945; Connell and Cross, 1950). However, this situation is by no means universal. On the South African, Peruvian, and California coasts, red water occurs during periods when precipitation is at a minimum. Furthermore, where such measurements have been made, the salinity in patches of red water does not appear to be significantly lower than that of the surrounding, clear ocean water (Ketchum and Keen, 1948; Chew, 1953; Torrey, 1902).

It is quite likely, however, that land drainage is important to the dinoflagellates from another aspect, that of providing their necessary organic matter. Whatever the function of these substances, it is possible that they are required in such minute concentrations as to satisfy the requirements of the organisms, although the entraining fresh-water may not appreciably dilute the sea water.

The concentration of dissolved nutrient salts in a given water mass prior to the onset of red water has not, to the author's knowledge, been determined. There are, to be sure, small, local occurrences which appear to be related to the introduction of domestic pollutants (Braarud, 1945; Connell and Cross, 1950). In these instances there are perhaps sufficient nutrients, as well as the necessary organic growth factors, to support populations approaching red water proportions. However, in the majority of red water occurrences there is no indication of an unusually high enrichment of the water prior to the outbreak.

Brongersma-Sanders (1948) emphasized that red water is associated with those regions of the world where upwelling brings deep, cold, nutrient-rich water to the surface (i.e., Southwest Africa, Peru, California). She also pointed out, however, that such upwelled water normally supports a luxuriant growth of diatoms, and that red water occurs in these regions only during the summer months when upwelling, and presumably the nutrient level, is greatly reduced. On the Indian coast, the southwest monsoon is associated with high enrichment and maximum productivity of the waters. Under these conditions it is again the diatoms which flourish, and red water appears only when the monsoon has ended.

Ketchum and Keen (1948) measured the total phosphorus content of sea water collected in and outside of patches of red water which occurred off the west coast of Florida in 1946-47. They found concentrations of total phosphorus in the red water (including that contained in the organisms) up to ten times as high as that of the clear water outside of the local patches. These authors emphasize the necessity for a mechanism either for accumulating these concentrations of phosphorus in the water prior to the growth of the organisms, or for concentrating the organisms after they have grown. There was no indication that the first of these alternatives was true.

The maximum concentration of phosphorus in the deep offshore waters of the Caribbean (2 microgram atoms per liter), as determined by Rakestraw and Smith (1937), does not approach the maximum observed in the red water patches (20 microgram atoms per liter), so upwelling could not have provided sufficient enrichment to be an important factor. It is also unlikely that land drainage could account for this phosphorus. Since the red water region contained only about 10% freshwater, runoff would have to carry tremendous concentrations of this element to account for the amount present in the organisms (estimated at 17,000 lb of pure phosphorus per square mile of red water). Chew (1953), who investigated a minor outbreak of red water in the same region in 1952, found that the lower salinity, coastal water, which contained the river drainage, had lower concentrations of phosphorus than the open waters of the Gulf of Mexico.

Thus it would appear that there may be insufficient nutrients normally present in sea water, and no known mechanism for concentrating them to a sufficiently high level, to support the development of a typical red tide. The remaining possibility, that the organisms themselves become concentrated after growth, will be discussed below.

In every case in which the vertical distribution of dinoflagellates in red water has been examined, it was found that the organisms were concentrated in relatively narrow bands usually at the surface of the water (Whitelegge, 1891; Nishikawa, 1901; Hirasaka, 1922; Martin and Nelson, 1929). In three instances of small, local outbreaks of red water, cell counts were made in a vertical column, revealing in each case that the maximum concentration of dinoflagellates occurred at the surface (Table III).

It is very doubtful that the organisms could have grown in such a pattern of distribution. As pointed out above, there would appear to be insufficient nutrients available to support such populations. Furthermore the high light intensities at the surface of the ocean are normally inhibitory to photosynthesis (Stanbury, 1931; Jenkin, 1937), particularly in the case of dinoflagellates, many of which appear to prefer reduced illumination (Barker, 1935; King, 1950). Hence, dense aggregations of dinoflagellates at the surface of the water must be

TABLE III  
Vertical Distribution of Dinoflagellates in Red Water  
(Figures indicate organisms per liter)

Depth (meters)	<i>Peridinium triquetrum</i>		<i>Gonyaulax monilata</i>
	Braarud (1945)	Marshall (1927)	Slobodkin (1953)
0	361,000	32-56,000	8,200,000
0.3			5,700,000
2.0	45,000	—	
2.6			450,000
4.0	11,000	—	
6.0	—	800	—

explained by concentrations of the organisms themselves, either by means of their active motility, or by passive floatation, should they become less dense than sea water.

Gran and Braarud (1935) observed that *Peridinium triquetrum* was often found in maximum concentrations at the surface, but that this organism could occur at any depth from 0 to 25 meters. They concluded that it could seek out the level at which the general conditions for growth were most favorable. Halse (1950) demonstrated marked vertical diurnal migrations in several dinoflagellates, the characteristics of which differed considerably from species to species. Thus *Ceratium fusus* and *C. tripos* rose to the surface at night and sank to the lower depths during the day, while *Gonyaulax polyedra* and *Proocentrum micans* showed the opposite response to light, rising in the daytime and sinking at night.

Concentration of dinoflagellates by means of such migrations may, at times, create red water conditions. Hirasaka (1922) observed red water in Gokasho Bay, Japan, in which the entire bay was apparently affected, in contrast to the usual situation in which red water is restricted to patches or streaks. According to this author, the organisms were confined to a band no more than 3 to 4 ft thick which appeared to migrate diurnally, the red color being most conspicuous in the late afternoon.

In many cases, however, the red water organisms remain at the surface of the water at all times. Woodcock (personal communication) collected samples of *Gymnodinium brevis* during the 1946-47 red tide off Florida and observed that the organisms remained at the

surface of the vessel at all times of the day and night. Ketchum (personal communication) observed the same behavior in *Noctiluca miliaris* collected during a bloom at Friday Harbor. Hornell and Nayudu (1923) and Nishikawa (1901) found that the red water organisms remained floating at the surface of a bottle apparently until they encysted; then they sank to the bottom.

Harvey (1917) and Ketchum (unpublished) studied the specific gravity of *Noctiluca* collected from patches of red water. In each case the organisms remained floating at the surface of the sea water if the latter was diluted with fresh water until the dilution approached 50%, beyond which they became suspended or sank. It must be emphasized, however, that these observations were made upon cells collected during typical red water conditions. It must not be assumed that such low densities are typical of *Noctiluca* at all times, and it cannot be so assumed if one subscribes to the theory that they accumulate at the surface after their growth. In this connection, the present author has observed a healthy culture of *Noctiluca* grown by Dr. L. Provasoli in which the organisms were evenly distributed throughout the medium.

Pratje (1921) has suggested that *Noctiluca* undergoes physiological changes in response to its environment, and that senescent, nondividing cells lose density and become buoyant in sea water. He subscribes to the theory suggested above that the concentration of these organisms at the surface does not result from active migration, but from floatation. Spoehr and Milner (1949) and others have demonstrated that *Chlorella* ceases to divide and stores fats when the nitrogen in its medium becomes limiting. Fat storage in the autotrophic dinoflagellates is common. Many species accumulate bright yellow or red oil droplets (Graham, 1951). *Noctiluca* apparently does not store fats, but its buoyancy is explained on the basis of the low specific gravity of its cell sap possibly through the accumulation of  $\text{NH}_4^+$  ions (see Krogh, 1939). Such mechanisms as these are adequate to account for the floatation of dinoflagellates. It remains to be demonstrated that changes occur in their metabolism or osmo-regulation such as to make them buoyant only at certain stages of their development.

The accumulation of buoyant organisms at the water surface would, of course, be enhanced in the absence of vertical mixing of the water,



which would tend to carry them down again into deeper layers. Harvey (1917) mentions that *Noctiluca* could not be observed at the surface during windy days. A re-examination of Table II will reveal that most of the occurrences of red water throughout the world were accompanied or preceded by periods of calm weather and smooth seas. Such conditions, together with the high temperatures which usually accompany them, may further stabilize the water through thermal stratification, providing additional resistance to the vertical mixing of the organisms.

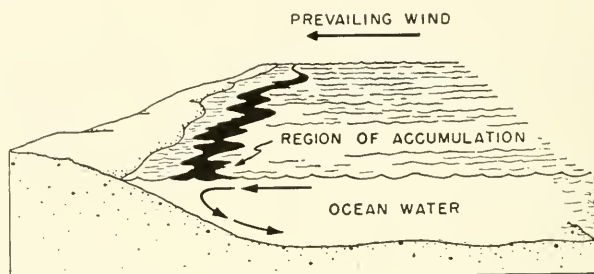


FIG. 3. Accumulation of floating material along shore by prevailing onshore wind.

Once the organisms have accumulated at the surface of the water, there are several means by which they may become further concentrated. Three such mechanisms will be discussed briefly below.

(1) Prevailing onshore winds: Surface water driven shoreward by prevailing onshore winds establishes a circular pattern, sinking at the waters edge and returning seaward at lower depths. Buoyant organisms will accumulate in windrows along shore or at the region of descent (Fig. 3).

(2) Where brackish coastal water, particularly in the vicinity of river mouths, meets open ocean water, there is a mixing and sinking of the two water masses along a line of convergence. Both types of water flow toward this line, and buoyant organisms will accumulate at or near the convergence line, producing streaks of floating material. (Fig. 4).

(3) Convection cells: Wind-driven vertical convection cells may be established which rotate alternately clockwise and counterclockwise



with their vertical axes perpendicular to the direction of the prevailing wind. Floating objects will accumulate in the region between the descending components of two such adjoining cells. Under these conditions parallel streaks of floating matter are produced (Fig. 5). (For a more detailed description of this process, see Langmuir, 1938; Stommel, 1949.)

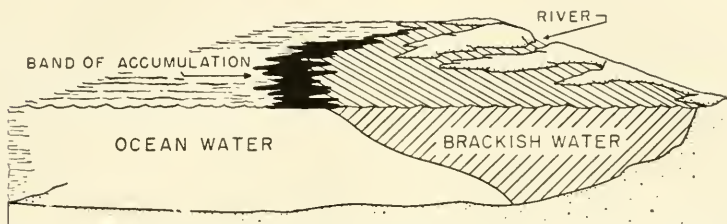


FIG. 4. Accumulation of floating material at convergence of water masses of different density.

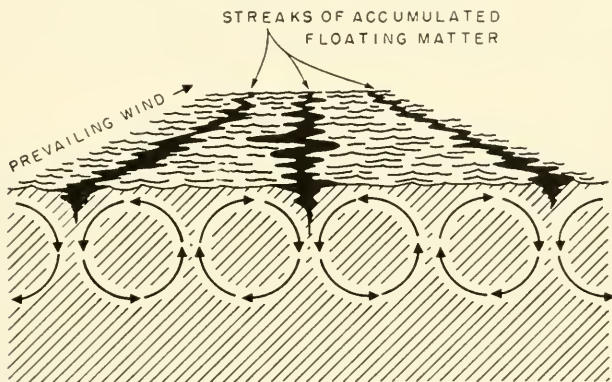


FIG. 5. Accumulation of floating material by wind-driven convection cells.

These and other similar mechanisms are undoubtedly important means of concentrating dinoflagellates to the extent to which they are observed in red water conditions. The existence of such processes is indicated by the repeated mention of patches or streaks of organisms in many descriptions of red water (see Table II). Bary (1953) has indicated that convection cells are responsible for the streaky distribution of floating masses of the ciliate *Cyclotrichium meunieri* in Wellington Harbor, New Zealand.

Thus through the combined processes of floatation and surface concentration, a method is possible by means of which red water conditions may be produced without the excessive enrichment of the water, and, indeed, without an unusually heavy growth of dinoflagellates. This may be illustrated by the following hypothetical situation.

According to Riley (1937, 1938) phosphate-phosphorus values for the surface waters of the Gulf of Mexico range from 0.02 to 0.5 microgram atom per liter. Since red water often occurs when nutrient levels are low, let us propose a situation in which the phosphate-phosphorus concentration is 0.05 microgram atom or  $1.55 \times 10^{-6}$  gram per liter. Let us further assume that other mineral nutrients, organic growth factors, etc., are present in the same or higher concentrations as phosphorus relative to the requirements of a given species of dinoflagellate.

No data are available concerning the dry weight and elementary analysis of red water organisms. However, these may be roughly estimated from known figures for other plankton organisms, and values may be obtained which are probably reliable within an order of magnitude.

*Gymnodinium brevis*, the dinoflagellate responsible for the Florida red tide of 1946-47, measures approximately 28 by 28 by 13 microns (Davis, 1948), and its volume may be roughly estimated at  $7.5 \times 10^{-9}$  cc. If it is assumed to have about the same density as sea water (at about 34‰ and 25° C in the coastal waters of the Gulf of Mexico), its wet weight will approximate  $7.7 \times 10^{-9}$  gram per cell.

Ketchum and Redfield (1949) found that the dry weight of a variety of planktonic algae was approximately 25% of their wet weight, and that their phosphorus content was rather constant at about 2.5% of the dry weight. Using these figures for *Gymnodinium* (which are probably somewhat high for dinoflagellates in general) a value of  $0.48 \times 10^{-10}$  gram of phosphorus per cell is obtained.

A dissolved phosphorus concentration of  $1.55 \times 10^{-6}$  gram per liter will then support a population of

$$\frac{1.55 \times 10^{-6}}{0.48 \times 10^{-10}} = 3.2 \times 10^4 \text{ cells per liter}$$

if all of the phosphorus were utilized and no other nutrient was limiting.

If the dinoflagellates in this hypothetical situation are also able to utilize all the nutrients in a water column 10 meters deep, and then, due to changes in their specific gravity, accumulate in the upper meter, their concentration at the surface will be in the range of  $3.2 \times 10^5$  cells per liter. The action of winds, convergence, or convection may then easily concentrate this surface layer by another factor of 10 or 20, producing concentrations of organisms typical of red water conditions, which range anywhere from  $10^6$  to  $10^8$  cells per liter.

Thus there is no necessity to postulate obscure factors which would account for a prodigious growth of dinoflagellates to explain red water. It is necessary only to have conditions favoring the growth and dominance of a moderately large population of a given species, and the proper hydrographic and meteorological conditions to permit the accumulation of organisms at the surface and to effect their further concentrations in localized areas.

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## DISCUSSION

Dr. Sweeney: Dr. Ryther has made clear in his presentation, an understanding of the nutritional requirements of the dinoflagellates is prerequisite to any explanation of the occurrence of "red tides." These organisms have been found to require for growth, substances not present in aged autoclaved sea water and usually supplied by the addition to the culture medium of small amounts of soil extract. In our laboratory, an analysis of the activity of soil extract with respect to the growth of bacteria-free *Gymnodinium splendens* has shown that soil extract may be replaced by vitamin B<sub>12</sub>, the optimum concentration being at 0.01 microgram vitamin B<sub>12</sub> per liter. Provasoli has shown that the dinoflagellate *Gyrodinium* sp. also requires vitamin B<sub>12</sub>. The organic requirements of *Prorocentrum micans* and *Gonyaulax polyedra* appear to be more complex and cannot be met by vitamin B<sub>12</sub> alone.

*Gonyaulax polyedra* may be grown successfully in mineral-enriched aged sea water with soil extract. Maximum populations reached are of the order of 40,000 cells per milliliter and the generation time is 40 to 60 hours. This organism is bioluminescent in culture. (*Prorocentrum micans*, *Gymnodinium splendens*, and *Gyrodinium* in our cultures are not luminescent.) Because it is both luminescent and photosynthetic, *Gonyaulax* provides interesting material for the study of bioluminescence.



## Bioluminescence in *Gonyaulax polyedra*\*

F. T. HAXO and BEATRICE M. SWEENEY  
Scripps Institution of Oceanography,  
University of California, La Jolla, California

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*Gonyaulax polyedra* is a photosynthetic marine dinoflagellate which is occasionally responsible for striking luminescent displays in the coastal waters of Southern California. The individual cells are armored; they are polyhedral in shape,  $45\mu$  long and  $41\mu$  wide and contain a heavily pigmented brown protoplast, in which the chlorophyll is masked by an abundance of carotenoid pigments.

The unialgal culture of *Gonyaulax polyedra* investigated was started from net samples collected off Scripps Pier on September 22, 1952. Since that time, the organism has been maintained in aged sea water, diluted to 75% of full strength with twice-distilled water, and supplemented with  $2 \times 10^{-3}M$   $KNO_3$ ,  $2 \times 10^{-4}M$   $K_2HPO_4$ ,  $6 \times 10^{-6}M$   $FeCl_3$ ,  $6 \times 10^{-7}M$   $MnCl_2$ , ethylene diamine tetracetic acid (EDTA), 10 mg per liter, and 2% soil extract. Liquid flask cultures were grown either at room temperature in a window with a northern exposure, or at  $20^\circ C$  under continuous illumination from white fluorescent lamps, at an intensity of about 700 foot-candles. Growth was about equally good in full-strength and half-strength sea water, or in media in which the supplementary salts were reduced to one-half the above values.

Soil extract was required for growth in aged sea water and could not be replaced by vitamin  $B_{12}$  alone. No growth, however, was obtained in artificial sea water supplemented with soil extract. At  $20^\circ C$ ,

\* Scripps Contribution No. 744.

maximum rate of growth was obtained at 500–800 foot-candles. The generation time was 56 hours (0.43 division in 24 hours), and the maximum cell densities were 30,000–50,000 cells per milliliter.

Photosynthesis of *Gonyaulax* cell suspensions was measured in artificial sea water (formula of Emerson and Green, 1934, modified to contain one-fourth the normal amount of Ca and Mg) by the direct manometric method. In saturating light (800 ft-c) at 20° C and non-limiting CO<sub>2</sub> concentration, as provided by 0.0234 M bicarbonate – 0.0016 M carbonate buffer, the rate of photosynthetic oxygen production was as follows (corrected for respiration): 350 mm<sup>3</sup> O<sub>2</sub>/hr/10<sup>6</sup> cells; 14,800 mm<sup>3</sup> O<sub>2</sub>/hr/g solids; 5000 mm<sup>3</sup> O<sub>2</sub>/hr/mg chlorophyll *a* plus *c*, estimated by the equations of Richards (1952). Under these conditions, the rate of oxygen production was about 5 times that of respiratory oxygen consumption. The temperature optimum for photosynthesis in *Gonyaulax* is about 30° C.

*Gonyaulax* has retained the ability to luminesce in culture for 1½ years. Light is emitted on mechanical stimulation as a bright bluish-green flash of short duration. Stimuli repeated at intervals of one minute elicit progressively weaker responses. Since preliminary observation with dark-adapted eyes indicated that luminescence of cell suspensions exposed to light was considerably weaker than those darkened for a few hours, a study was undertaken of the effect of light on the luminescence of this organism. The apparatus employed for measuring and recording light emission included a photomultiplier tube (931A) and appropriate amplifying circuit, with variable sensitivity, coupled to a Speedomax recorder. Insertion of a condenser in the circuit served to slow down the instrumental response and to provide smooth curves for convenience in planimetric calculations of total light emission.\* Aliquot cell suspensions of 3 ml were taken from mature cultures (20,000–40,000 cells per milliliter) grown at 20° C in constant light of 700 ft-c. The aliquots were held in the lighted incubator for 4 hours and then placed in darkness. Measurements of light emission by different aliquots were made at intervals during the dark adaptation period. Luminescence, stimulated by passing a stream of air through the cell suspension at a constant rate of

\* This apparatus was designed by Mr. James Snodgrass, Division of Special Developments, Scripps Institution of Oceanography.

flow for one minute, rose sharply to a maximum in less than 7 seconds (less than a second when measured with a faster recording device) and decreased to a low steady state.

Cell suspensions taken directly from the lighted incubator luminesced only slightly on stimulation. When such cells were placed in

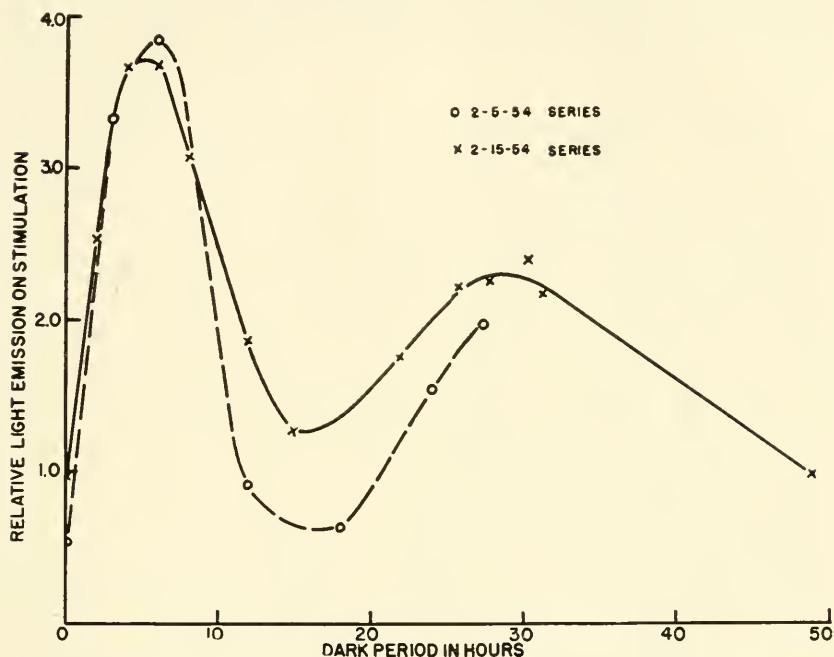


FIG. 1. Variation in luminescence of *Gonyaulax polyedra* cell suspensions with time in darkness, after growth in white light at 700 ft-c. Each measurement represents the integrated light emission during mechanical stimulation of one minute duration by different aliquots, not otherwise agitated until the indicated time.

darkness, the capacity for luminescence increased with time in darkness and reached a peak after 6–8 hours, as shown in Fig. 1. With the time in darkness further increased, luminescence decreased progressively, reaching a low value after 15–18 hours. This decline was followed by a second increase in light emission, which reached a peak after 28–30 hours in darkness. The second peak was lower than the first and was followed by a second decrease in luminescence. This

fluctuation pattern in luminescence has been observed a number of times and appears to be independent of the time of day at which cultures are placed in darkness. Instrumental fluctuations and variations between duplicates were of the order of 2-4% and could not account for the changes observed.

Cell suspensions placed in white light after an optimum dark adaptation time (5-6 hr) showed a progressive decrease in luminescence

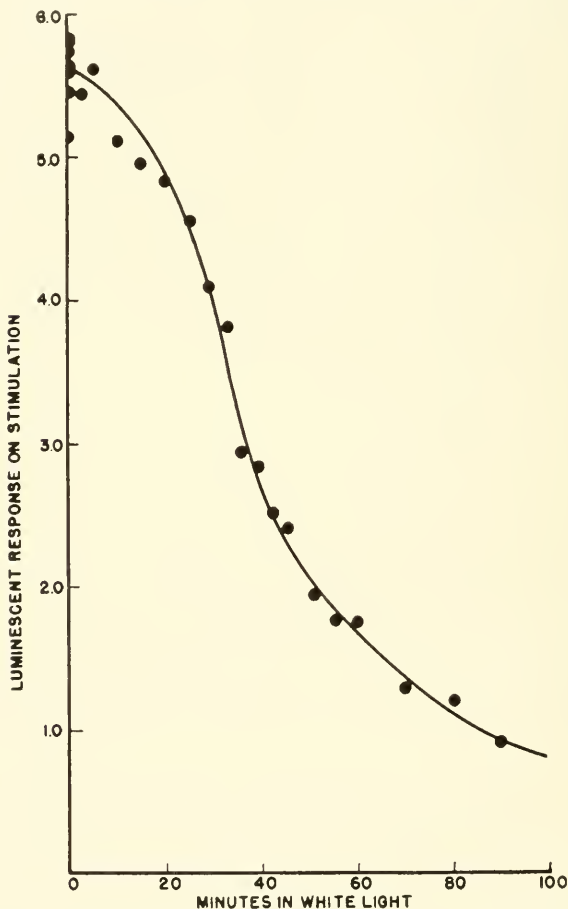


FIG. 2. Decrease in luminescence of *Gonyaulax polyedra* suspensions upon exposure of dark-adapted cells to 700 ft-c white light. The cells were grown in the light and placed in darkness for 6 hours prior to irradiation. Relative light emission prior to dark adaptation was 0.75.



with time of irradiation (Fig. 2). When exposed to fluorescent light of 700 ft-c intensity, maximum inhibition of luminescence was obtained in 90 minutes, the final level of light emission being about equal to that of cultures maintained continuously in the light. Preliminary experiments with broad band pass filters intercepting the fluorescent light indicate that the blue-violet end of the spectrum is the most effective in suppressing luminescence. There is a suggestion of a small effect of red light, and a more detailed determination of the effectiveness spectrum is planned.

The inhibition of luminescence by light in *Gonyaulax* suggests a photooxidation of one of the components of the luminescent system, as in luminous bacteria and *Cypridina* extracts. The observation that the maximum light emission of dark-adapted cultures increases with the duration of the previous photosynthetic period implies that light exerts a second effect on luminescence, probably through the building up of photosynthetic products or intermediates necessary for the maintenance of the luminescent system. The decline in luminescence capacity in the dark observed during the period 8–18 hours (Fig. 1) may be interpreted as a progressive depletion of these substances. It has been suggested that, as starvation proceeds, degenerative processes within the cell result in the release of additional reserves and lead to the second peak observed in darkened cells. The final decline in luminescence after 30 hours is associated with loss in cell viability and death. This undoubtedly results from starvation, since *Gonyaulax* is nutritionally dependent on photosynthesis.

The above findings recall earlier reports (cf. Harvey, 1952) based on visual observations that natural populations of dinoflagellates show fluctuations in luminescence when brought into the laboratory. In unialgal cultures of *Gonyaulax polyedra* maintained in the laboratory, luminescence is partially suppressed by exposure to bright light. The ability to luminesce on stimulation is enhanced in darkened cultures, and subsequently falls and rises before the cells die of starvation.

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